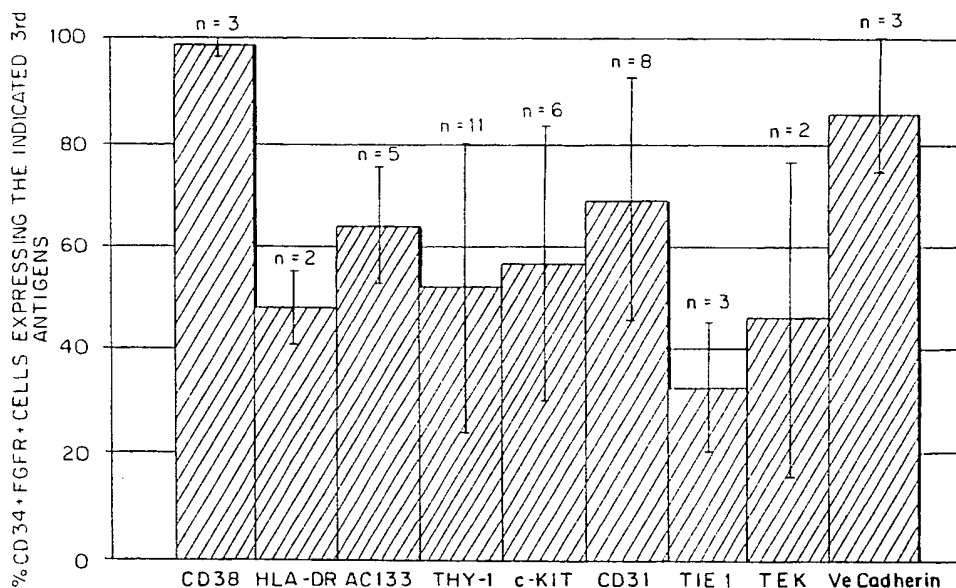




## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12N 5/08, A61K 35/14, 35/28, A61P 43/00</b>		<b>A3</b>	(11) International Publication Number: <b>WO 00/12683</b> (43) International Publication Date: 9 March 2000 (09.03.00)
(21) International Application Number: PCT/US99/19730 (22) International Filing Date: 31 August 1999 (31.08.99) (30) Priority Data: 60/098,480 31 August 1998 (31.08.98) US (71) Applicant: NEW YORK UNIVERSITY [US/US]; 70 Washington Square South, New York, NY 10012 (US). (72) Inventors: WILSON, E., Lynette; Apartment 5D, 447 East 65th Street, New York, NY 10021 (US). BURGER, Patricia, E.; 9 Spanish Steps Ferguson Road, Bloubergstrand 7441, Cape Town (ZA). (74) Agent: BROWDY, Roger, L.; Browdy and Neimark, P.L.L.C., Suite 300, 419 Seventh Street N.W., Washington, DC 20004 (US).			(81) Designated States: AU, CA, IL, JP, ZA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published With international search report.  (88) Date of publication of the international search report: 29 June 2000 (29.06.00)

(54) Title: STEM CELLS BEARING AN FGF RECEPTOR ON THE CELL SURFACE



## (57) Abstract

A composition of substantially purified pluripotent stem cells are positive both for fibroblast growth factor receptor (FGFR) and a phenotype indicative of a primitive state, such as CD34<sup>+</sup>, CD34<sup>-lin</sup><sup>-</sup>, Thy-1<sup>+</sup>, AC133<sup>+</sup> or c-kit<sup>+</sup>. The state of being an embryonic stem cell is also a phenotype indicative of a primitive state. This population may be further defined by the subpopulations thereof which have another marker thereon indicative of endothelial cells, such as TIE-1<sup>+</sup>, TEK<sup>+</sup>, CD31<sup>+</sup>, VE-Cadherin<sup>+</sup> or VEGFR<sup>+</sup> or indicative of stromal cells, such as STRO-1<sup>+</sup>.

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## INTERNATIONAL SEARCH REPORT

Int. Application No.

PCT/US 99/19730

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/08 A61K35/14 A61K35/28 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDWINE J M ET AL: "In situ expression of fibroblast growth factor receptors by oligodendrocyte progenitors and oligodendrocytes in adult mouse central nervous system." JOURNAL OF NEUROSCIENCE RESEARCH, (1997 OCT 15) 50 (2) 229-37. , XP000891229 the whole document	1-28
A	RATAJCZAK MARIUSZ Z ET AL: "Effect of basic (FGF-2) and acidic (FGF-1) fibroblast growth factors on early haemopoietic cell development." BRITISH JOURNAL OF HAEMATOLOGY 1996, vol. 93, no. 4, 1996, pages 772-782, XP000891218 ISSN: 0007-1048 the whole document	1-28
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Date of the actual completion of the international search

20 March 2000

Date of mailing of the international search report

04/04/2000

Name and mailing address of the ISA

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# INTERNATIONAL SEARCH REPORT

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## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 690 926 A (HOGAM B.L.M.) 25 November 1997 (1997-11-25) the whole document	1-28
P,X	BURGER P E ET AL: "Isolation and characterization of a CD34+ population that expresses fibroblast growth factor receptors." BLOOD, 40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY; MIAMI BEACH, FLORIDA, USA; DECEMBER 4-8, 1998, vol. 92, no. 10 SUPPL. 1 PART 1-2, 15 November 1998 (1998-11-15), page 56A XP000891230 ISSN: 0006-4971 the whole document	1-28

## INTERNATIONAL SEARCH REPORT

Information on patent family members

In Application No

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5690926 A	25-11-1997	US 5453357 A	26-09-1995
		US 5670372 A	23-09-1997



STEM CELLS BEARING AN FGF RECEPTOR  
ON THE CELL SURFACE

FIELD OF INVENTION

5           The present invention is directed to a new phenotype of stem cells which contain a fibroblast growth factor receptor (FGFR) on the cell surface thereof and further have a phenotype indicative of a primitive state. The present invention is further directed to subpopulations thereof having a phenotype  
10 indicative of endothelial or stromal cells.

BACKGROUND OF THE INVENTION

          The ability of tissues and organs to develop, remodel, regenerate, and repair depends on the existence of stem cells (also known as progenitor cells) that, upon division, form more  
15 differentiated progeny. Stem cells have been found in the epidermis, the intestinal epithelium, and the hematopoietic system. There is mostly indirect evidence of stem cells in mesenchymal tissues. *In vivo* and *in vitro* studies have provided evidence of osteogenic precursor cells in bone marrow and other  
20 stromal cell preparations. However, the identity of cells in these tissues and their relationship to cells with classical stem cell characteristics have yet to be established.

          Endothelial cells are part of the normal bone marrow stroma. Long-term cultures of human bone marrow contain a  
25 complex mix of stromal cells including adipocytes, fibroblasts, endothelial cells, macrophages, and smooth muscle cells. Endothelial cells and hematopoietic cells are thought to be derived from the common progenitor cells, hemangioblasts.

30           Cell surface molecules on various types of cells, and particularly hematopoietic cells, are given a cluster of differentiation (CD) designation in which each CD molecule designation describes a surface molecule (marker) identifiable by a cluster of monoclonal antibodies that display the same  
35 cellular reactivity. CD designations are assigned at regularly held international workshops on human leukocyte differentiation antigens. For example, the CD19 marker is

specific to B cells, and the CD33 marker is specific to myeloid cells. At the present time, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker which has been indicated as  
5 being present on stem cells is CD34. However, this marker is also found on a significant number of lineage-committed progenitors. Other markers which are known or thought to be present primarily on stem cells, i.e., "primitive" markers, include AC133 (Yin et al, 1997; Buhning et al, 1999), Thy-1  
10 (Murray et al, 1995) and c-kit (D'Arena et al, 1998).

It is known that a small number of circulating CD34<sup>+</sup> hematopoietic stem cells are present in peripheral blood. As the major source of CD34<sup>+</sup> hematopoietic stem cells in the adult is the bone marrow, the purpose of this small, circulating  
15 CD34<sup>+</sup> cell population is unknown. One explanation is that the bone marrow is "leaky", and the stem cells escape, circulate and return to the marrow. A second possibility is that the function of these circulating stem cells is to seed sites, such as the liver and the spleen, which can function as  
20 additional sites of hematopoiesis in a crisis.

The human CD34<sup>+</sup> hematopoietic population isolated from bone marrow, cord blood, and peripheral blood is a heterogeneous population that contains hematopoietic stem cells. Recent evidence indicates that circulating CD34<sup>+</sup> cells  
25 also contain endothelial stem cells, which may also circulate (Asahara et al, 1997; Nieda et al, 1997; Shi et al, 1998; Lin et al, 1998). Asahara et al (1997) have shown that CD34<sup>+</sup> cells isolated from the peripheral blood can be incorporated into the endothelium of ischaemic blood vessels of recipient  
30 animals. Purified umbilical cord blood CD34<sup>+</sup> cells also give rise to von Willebrand factor-expressing endothelial cells *in vitro*, providing additional evidence for a circulating progenitor endothelial cell (Nieda et al, 1997). In addition, bone marrow derived CD34<sup>+</sup> cells also contain a transplantable  
35 stromal stem cell (Prockop, 1997; Pereira et al, 1998).



Recently, convincing evidence has been presented (Goan et al, 1997) that human CD34<sup>+</sup> progenitor cells from peripheral blood or cord blood that were transplanted into NOD/SCID immunodeficient mice gave rise to human stromal cells. The human stromal cells expressed the endothelial cell-specific vascular endothelial growth factor (VEGF) receptor-2 (KDR) and von Willebrand factor, indicating that they were of endothelial origin. There is also recent evidence that infusion of whole bone marrow cells into recipient mice results in fibroblasts of donor origin in a number of non-hematopoietic tissues (Prockop, 1997; Pereira, 1998), indicating that stromal progenitor cells reside in the bone marrow. As CD34 has been shown to be expressed by bone marrow stromal precursor cells (Simmons et al, 1991), it is possible that these stromal progenitors reside in the bone marrow within the CD34<sup>+</sup> progenitor population.

The CD34<sup>+</sup> progenitor population is, therefore, a heterogeneous fraction that may include precursor cells of the hematopoietic, endothelial, and stromal/fibroblast lineages. In addition, pluripotent mesenchymal stem cells capable of differentiating into cells of the osteogenic, chondrogenic, tendonogenic, adipogenic and myogenic lineages have been shown to reside within the bone marrow microenvironment (Majumdar et al, 1998). There is recent literature indicating that circulating endothelial progenitor/stem cells exist, and that stromal stem cells in marrow serve as a source for continual renewal of cells in a number of non-hematopoietic tissues. A common embryological precursor that gives rise to both hematopoietic and endothelial cells has recently been identified (Suda et al, 1997; Choi et al, 1998; Caprioli et al, 1998).

Recent evidence has also shown that embryonic stem (ES) cells can give rise to endothelial cells (Hirashima et al, 1999).

Fibroblast growth factors (FGFs) can synergize with other factors to stimulate hematopoietic progenitor cell

proliferation (Wilson et al, 1991; Quito et al, 1996; Allouche, 1995; Yuen et al, 1998, U.S. Patent 5,612,211; U.S. Patent 5,817,773). It has also been shown that basic FGF (FGF-2) acts to antagonize cytokines that induce  
5 differentiation (Burger et al, 1994). In addition, low amounts of FGF-2, on the order of 10-100 pg/ml, induce a more primitive phenotype in human K562 leukemic cells.

It would be very useful to be able to isolate stem cells which are progenitors of endothelial and/or stromal  
10 cells. The more primitive the stem cell, the more useful it is in bone marrow transplantation. Furthermore, endothelial stem cells and stromal stem cells, or a stem cell which is a progenitor of both, would find many utilities in repairing damaged vasculature and in treating other conditions where  
15 endothelial or stromal cells need to be replenished.

#### SUMMARY OF THE INVENTION

It is an object of the present invention to overcome the deficiencies in the prior art.

It is another object of the present invention to  
20 isolate a new phenotype of stem cells.

It is a further object of the present invention to identify and isolate stem cells for use in stem cell transplantation.

It is yet another object of the present invention to  
25 identify and isolate endothelial and/or stromal stem cells.

A small population of cells having a "primitive phenotype", such as CD34<sup>+</sup> or CD34<sup>+</sup>lin<sup>-</sup>, has been isolated which express cell surface receptors for fibroblast growth factor (FGF). The population of cells bearing FGF receptors (FGFR)  
30 are designated as FGFR<sup>+</sup>. The FGFR<sup>+</sup> primitive phenotype cell population has several unique properties:

(1) The CD34<sup>+</sup>FGFR<sup>+</sup> cells are predominantly present in the region of the fluorescence-activated cell sorter profile having low forward scatter (FSC) and low side scatter  
35 (SSC). Thus, the majority of the cells of the population are very small and of low granularity. These small cells are

located in the FSC/SSC region of the fluorescence-activated cell sorter profile that is normally not analyzed, as this area contains many of the dead and apoptotic cells.

Interestingly, this region has recently been shown to be the site of a mesenchymal stem cell population (Zohar et al, 1997). The CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells have FSC/SSC properties that are similar to those of the CD34<sup>+</sup>FGFR<sup>+</sup> cells. This CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup> population also includes significant numbers of cells with higher FSC properties.

(2) The CD34<sup>+</sup>FGFR<sup>+</sup> cells are deeply dormant, which is characteristic of a stem cell population. They do not proliferate in culture until 30-60 days after isolation.

The FGFR<sup>+</sup> primitive phenotype cell population is a unique stem cell population that is a precursor cell for endothelium and/or hematopoiesis and/or stroma. The FGFR<sup>+</sup> primitive phenotype cell population, obtained either from general circulation, the bone marrow, cord blood or embryonic cells, is capable of forming endothelial, blood and stromal cells, depending on the need at the time.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a bar graph summarizing the fourteen experiments of Example 1 and showing the percent of CD34<sup>+</sup>FGFR<sup>+</sup> cells which express the indicated third antigens.

Figures 2A-2F are flow cytometry plots for Experiment 7 of Example 1. Figure 2A is a dot plot of FSC versus SSC of all events with region R1 drawn to eliminate the area containing most of the cell debris and doublets. Figure 2B is a histogram showing the intensity of staining with the dye 7-aminoactinomycin D (7-AAD). The region R2 is drawn to delineate live cells. Figure 2C is a dot plot of SSC versus CD34 gated on R1 AND R2. The region R3 is drawn to delineate CD34<sup>+</sup> cells. Figure 2D is a dot plot of FSC versus SSC gated on R1 AND R2 AND R3, thus showing the FSC/SSC characteristics of live CD34<sup>+</sup> cells in R1. Figure 2E is a dot plot of CD34 versus FGFR gated on R1 AND R2. The region R4 is drawn to delineate CD34<sup>+</sup>FGFR<sup>+</sup>. Figure 2F is a dot plot of FSC versus

SSC gated on R1 AND R2 AND R4, thereby showing the characteristics of live CD34<sup>+</sup>FGFR<sup>+</sup> cells in R1.

Figure 3 is a bar graph showing the percent of live FGFR<sup>+</sup> cells co-expressing the indicated antigens.

5           Figure 4 is a bar graph showing the growth of various cell populations in the presence or absence of FGF-2 or a combination of FGF-2 plus VEGF.

#### DETAILED DESCRIPTION OF THE INVENTION

Stem cells, when transplanted, can restore the  
10 production of hematopoietic, endothelial and stromal cells to a patient who has lost such production due to, for example, radiation therapy. By isolating FGFR<sup>+</sup> primitive phenotype cells, preferably CD34<sup>+</sup>FGFR<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>, from other cells in the body, it is possible to obtain relatively pure stem  
15 cells, preferably separate from contaminating cells and other substances, so that the stem cells can be safely transplanted into a patient in need thereof.

The unique isolated cells of the present invention are separated from other cells by virtue of their CD34<sup>+</sup> or  
20 CD34<sup>-</sup>lin<sup>-</sup> state and possession of fibroblast growth factor receptors. The cells can be isolated by conventional techniques for separating cells, such as those described in Civin, U.S. Patents 4,714,680, 4,965,204, 5,035,994, and 5,130,144, Tsukamoto et al 5,750,397, and Loken et al, U.S.  
25 Patent 5,137,809, all of which are hereby incorporated by reference in their entirety. Thus, for example, a CD34-specific monoclonal antibody or an FGFR-specific antibody can be immobilized, such as on a column or on magnetic beads. The entire cell population may then be passed through the column  
30 or added to the magnetic beads. Those which remain attached to the column or are attached to the magnetic beads, which may then be separated magnetically, are those cells which contain a marker which is recognized by the antibody used. Thus, if the anti-CD34 antibody is used, then the resulting population  
35 will be greatly enriched in CD34<sup>+</sup> cells. If the antibody used is FGFR, then the resulting population will be greatly

enriched in FGFR<sup>+</sup> cells. That population may then be enriched in the other marker by repeating the steps using a solid phase having attached thereto an antibody to the other marker.

Another way to sort CD34<sup>+</sup>FGFR<sup>+</sup> cells is by means of  
5 flow cytometry, most preferably by means of a fluorescence-activated cell sorter (FACS), such as those manufactured by Becton-Dickinson under the names FACScan or FACSCalibur. By means of this technique, the cells having a CD34 marker thereon are tagged with a particular fluorescent dye by means  
10 of an anti-CD34 antibody which has been conjugated to such a dye. Similarly, the FGFR marker of the cells are tagged with a different fluorescent dye by means of an anti-FGFR antibody which is conjugated to the other dye. When the stained cells are placed on the instrument, a stream of cells is directed  
15 through an argon laser beam that excites the fluorochrome to emit light. This emitted light is detected by a photomultiplier tube (PMT) specific for the emission wavelength of the fluorochrome by virtue of a set of optical filters. The signal detected by the PMT is amplified in its own channel and  
20 displayed by a computer in a variety of different forms—e.g., a histogram, dot display, or contour display. Thus, fluorescent cells which emit at one wavelength, express a molecule that is reactive with the specific fluorochrome-labeled reagent, whereas non-fluorescent cells or fluorescent  
25 cells which emit at a different wavelength do not express this molecule but may express the molecule which is reactive with the fluorochrome-labeled reagent which fluoresces at the other wavelength. The flow cytometer is also semi-quantitative in that it displays the amount of fluorescence (fluorescence  
30 intensity) expressed by the cell. This correlates, in a relative sense, to the number of the molecules expressed by the cell.

Flow cytometers are also equipped to measure non-fluorescent parameters, such as cell volume or light scattered  
35 by the cell as it passes through the laser beam. Cell volume is usually a direct measurement. The light scatter PMTs

detect light scattered by the cell either in a forward angle (forward scatter; FSC) or at a right angle (side scatter; SSC). FSC is usually an index of size, whereas SSC is an index of cellular complexity, although both parameters can be  
5 influenced by other factors.

Preferably, the flow cytometer is equipped with more than one PMT emission detector. The additional PMTs may detect other emission wavelengths, allowing simultaneous detection of more than one fluorochrome, each in individual  
10 separate channels. Computers allow the analysis of each channel or the correlation of each parameter with another. Fluorochromes which are typically used with FACS machines include fluorescein isothiocyanate (FITC), which has an emission peak at 525 nm (green), R-phycoerythrin (PE), which  
15 has an emission peak at 575 nm (orange-red), propidium iodide (PI), which has an emission peak at 620 nm (red), 7-aminoactinomycin D (7-AAD), which has an emission peak at 660 nm (red), R-phycoerythrin Cy5 (RPE-Cy5), which has an emission peak at 670 nm (red), and allophycocyanin (APC), which has an  
20 emission peak at 655-750 nm (deep red).

These and other types of FACS machines may have the additional capability to physically separate the various fractions by deflecting the cells of different properties into different containers.

25 Any other method for isolating the CD34<sup>+</sup>FGFR<sup>+</sup> population of a starting material, such as bone marrow, peripheral blood or cord blood, may also be used in accordance with the present invention. The various subpopulations of the present invention may be isolated in similar manners.

30 The isolated cell population of this invention can be used in therapeutic methods, such as stem cell transplantation, as well as other therapeutic methods as described below, as well as others that are readily apparent to those skilled in the art. For example, the isolated cell  
35 populations can be administered directly by intravenous route to a mammalian patient requiring a bone marrow transplant in

an amount sufficient to reconstitute the patient's hematopoietic and immune system. Precise, effective quantities can be readily determined by those skilled in the art and will depend, of course, upon the exact condition being  
5 treated by the therapy. In many applications, however, an amount containing approximately the same number of stem cells found in one-half to one liter of aspirated marrow should be adequate.

Thus, a suspension of human cells from marrow or  
10 blood comprising cells which are positive both for CD34 and for fibroblast growth factor receptors, preferably substantially free of cells that are not positive for both CD34 and fibroblast growth factor receptors, can restore the production of hematopoietic cells to a human lacking  
15 production of these cells. A suspension of these isolated cells is administered to a patient in need thereof in an effective amount to restore production of hematopoietic/endothelial/stromal cells.

The patients in need of this product are those with  
20 a specific requirement for hematopoietic, endothelial or stromal cells. For example, patients with vascular injury, persons with genetic defects in their hematopoietic, stromal or endothelial cells, such as collagen deficiency, adenosine deaminase deficiency, or clotting factor deficiency. It is  
25 expected that the circulating stem cells will selectively home to sites of hematopoietic, endothelial or stromal cell damage/deficiency.

Other "primitive" phenotype indicators besides CD34<sup>+</sup> are also known. These include AC133<sup>+</sup> (Buhring et al, 1994; Yin  
30 et al, 1997), Thy-1 (Murray et al, 1995) and c-kit<sup>+</sup> (Buhring et al, 1994; D'Arena et al, 1998). Another population of primitive cells is CD34<sup>-</sup>lin<sup>-</sup>. The present inventors have discovered that the CD34<sup>+</sup>FGFR<sup>+</sup> population of the preferred embodiment of the present invention contains significant  
35 amounts of the AC133 marker (approximately 64%), the Thy-1

marker (approximately 52%) or the c-kit marker (approximately 57%). Many of these cells have more than one primitive marker.

Furthermore, certain markers are known to be  
5 endothelial markers. These include VE-Cadherin (also known as CD144) (Vittet et al, 1996), TIE-1 (also known as TIE) (Suda et al, 1997), TEK (also known as TIE-2) (Suda et al, 1997; Hamaguchi et al, 1999) and CD31 (also known as PECAM) (Watt et al, 1993). Significant quantities of each of these markers  
10 were also found on the CD34<sup>+</sup>FGFR<sup>+</sup> population. About 86% of this population co-expresses VE-Cadherin, about 70% co-expresses CD31; about 47% co-expresses TEK; and about 33% co-expresses TIE-1. This data indicates that the CD34<sup>+</sup>FGFR<sup>+</sup> population includes a primitive population of cells which are  
15 precursors of endothelial cells. The subpopulation with these endothelial markers can be isolated and are also part of the present invention. Certain additional markers are known to be stromal cell markers, such as STRO-1 (Gronthos et al, 1994). A subpopulation of the CD34<sup>+</sup>FGFR<sup>+</sup> cells of the present  
20 invention which is also STRO-1<sup>+</sup> is a primitive population of cells which are precursors of stromal cells. The subpopulation with these stromal markers can be isolated and are also part of the present invention.

The results as to co-expression of additional  
25 markers were obtained from fluorescence-activated cell sorter (FACS) analysis using specific antibodies to cell surface antigens. The antibodies were labeled with three or four different fluorochromes. The results show that 100% of live FGFR<sup>+</sup>Thy-1<sup>+</sup> cells co-express VE-Cadherin, 97% of live  
30 FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express VE-Cadherin, 91% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express Thy-1, and 67% of live FGFR<sup>+</sup>TEK<sup>+</sup> cells co-express Thy-1. Cell sorter systems using additional fluorochromes will be able to allow the separation of those cells in the CD34<sup>+</sup>FGFR<sup>+</sup> population which also express two or  
35 more of the various other primitive or endothelial markers discussed above. Similarly, other markers, such as the



vascular endothelial growth factor-receptor (VEGF-R) (also known as KDR), which is a marker for endothelial cells, can also be included in such analyses. It is predicted that a subpopulation of CD34<sup>+</sup>FGFR<sup>+</sup>Thy-1<sup>+</sup>VEGF-R<sup>+</sup> cells exist, which  
5 subpopulation can be identified and isolated by such systems by one of ordinary skill in the art without undue experimentation. This subpopulation represents a progenitor population capable of developing into either or both of the hematopoietic and endothelial lineages.

10 The isolated CD34<sup>+</sup>FGFR<sup>+</sup> cells grow exceedingly slowly in culture with a long lag of 4-6 weeks. The cells grow in an FGF-dependent manner, as shown in Example 3 and Table 5. A long dormant period is associated with a stem cell phenotype, indicating that these cells have growth characteristics  
15 compatible with stem cells.

While the CD34<sup>+</sup>FGFR<sup>+</sup> cells can be isolated in substantial purity, i.e., in a substantially homogeneous population, by the methods discussed above, such as, for example, by means of the FACS apparatus, it is not always  
20 necessary that the CD34<sup>+</sup>FGFR<sup>+</sup> stem cell population of the present invention be present in substantial purity. For example, for most purposes, it is sufficient if the population of cells contains greater than 90% of human stem cells characterized as CD34<sup>+</sup> and FGFR<sup>+</sup> or FGFR<sup>+</sup> with another  
25 indication of primitive phenotype. Other aspects of the present invention include subpopulations of the FGFR<sup>+</sup> primitive phenotype population which are substantially homogeneous for other markers. This includes the subpopulation of the FGFR<sup>+</sup> primitive phenotype human stem cells which are also positive  
30 for any one or more of the endothelial markers, any one or more additional primitive markers and/or any one or more of stromal cell markers, which subpopulation is substantially homogeneous or is a composition wherein greater than 90% of said cells are FGFR<sup>+</sup> primitive phenotype and positive for one  
35 or more of those additional markers. Thus, the subpopulation may be a substantially homogeneous population or a composition

in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and TIE-1<sup>+</sup>. The subpopulation may also be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and CD31<sup>+</sup> and/or TEK<sup>+</sup> and/or VEGFR<sup>+</sup> and/or VE-Cadherin<sup>+</sup> and/or positive for any other endothelial marker. Similarly, the subpopulation may be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and positive for one or more of the other primitive markers, such as AC133, Thy-1 and c-kit. The subpopulation may also be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and also positive for one or more of the other primitive markers and further positive for one or more of the endothelial markers. The subpopulation may also be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and also positive for one or more of stromal markers, such as CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and STRO-1<sup>+</sup>. Such subpopulations are also contemplated by the present invention.

With some of the utilities of the present invention, such as, for example, bone marrow transplantation, the stem cell population may be a substantially smaller percent of the total cell count being administered. The remaining cells may be filler cells, which may be cells incapable of replicating. Alternatively, the remaining cells may be any of the other types of cells from which the cells of the present invention are originally separated. Thus, for example, the present invention also comprehends populations containing at least 20% of any of the phenotypes of the present invention, i.e., CD34<sup>+</sup>FGFR<sup>+</sup>, CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>Thy-1<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>TIE-1<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>CD31<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>VEGF-R<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>Thy-1<sup>+</sup>VEGF-R<sup>+</sup>, etc. Such a low purity subpopulation still defines over the prior art and yet maintains many of the advantages of the present

invention for many of its proposed utilities. Compositions having greater than 30%, 40%, 50%, 60%, 70% or 80% of cells of any of the phenotypes of the present inventions are also considered to be part of the present invention.

5 Another way of defining the cellular compositions of the present invention is as a suspension of human cells, comprising pluripotent stem cells or endothelial stem cells which are substantially free of mature lymphoid and myeloid cells. Cells substantially all of which are of the FGFR<sup>+</sup>  
10 primitive phenotype are substantially free of mature lymphoid and myeloid cells.

Although the present invention has thus far been primarily described with respect to the preferred embodiment of CD34<sup>+</sup>FGFR<sup>+</sup> cells, it should be understood that FGFR<sup>+</sup> cells  
15 having other indications of primitive phenotype are also contemplated in accordance with the present invention. For example, CD34<sup>-</sup> cells which are also negative for lineage markers (lin<sup>-</sup>) may be even more primitive than CD34<sup>+</sup> cells (see Zanjani et al, 1999). Thus, the CD34<sup>-</sup>lin<sup>-</sup> phenotype is also  
20 considered to be an indication of primitiveness in accordance with the present invention. Additionally, when embryonic stem cells are used as the source of cells from which the population of the present invention is to be separated, all such cells, by definition, have a primitive phenotype. Thus,  
25 FGFR<sup>+</sup> cells separated from an embryonic stem cell source will inherently be FGFR<sup>+</sup> cells with a primitive phenotype. Furthermore, other primitive markers, such as AC133, Thy-1 and c-kit, may also be used as markers for primitiveness. Thus, in its broadest aspect, the present invention relates to  
30 phenotypes which are FGFR<sup>+</sup>, as well as positive for any phenotype indicating primitive state cells, including, but not limited to, CD34<sup>+</sup>, CD34<sup>-</sup>lin<sup>-</sup>, being embryonic stem cells, AC133<sup>+</sup>, Thy-1<sup>+</sup> and c-kit<sup>+</sup>. The present invention further relates to subpopulations thereof as described above.

35 The pluripotent FGFR<sup>+</sup> primitive phenotype stem cells, or pluripotent stem cells of any of the other phenotypes of

the present invention, have considerable commercial use, including one or more of the following:

(1) For bone marrow transplantation.

(2) To target delivery of anti-tumor agents.

5 Endothelial stem cells of the present invention can be used to target the delivery of angiostatic agents and anti-tumor agents to the rapidly proliferating vascular bed associated with tumors. Endothelial cells are long-lived, and the stem cells can be used as vectors to deliver angiostatic/antitumor  
10 agents to the rapidly expanding vascular bed associated with tumors without affecting the stable endothelium of established blood vessels.

(3) To coat valves and devices used in surgical procedures. The endothelial stem cells of the present  
15 invention can be used to coat valves and implant devices, eliminating many of the clotting problems currently associated with these devices. The endothelial stem cells can be cultured from specific individuals so that valves, implant devices, etc., may be coated with autologous endothelial  
20 cells. Panels of HLA-matched endothelial stem cells can also be produced for these purposes.

(4) As vectors for genetic engineering. Genetically engineered stem cells "homing" to the endothelium, bone marrow, or connective tissue stroma are long-lived and  
25 can secrete proteins, such as adenosine deaminase or clotting factors, as well as other proteins, such as t-PA, which promote thrombolysis. A wild-type gene can be incorporated into the endothelial stem cells, either by homologous or random recombination. With allogeneic endothelial stem cells,  
30 normal cells lacking the genetic defect can be used therapeutically. Other indications for gene therapy are introduction of drug resistance genes to enable normal stem cells to have an advantage and be subject to selective pressure, e.g., the multiple drug resistance gene. Disease  
35 other than those associated with endothelial cells may also be treated, where the disease is related to the lack of a

particular excreted product, such as a hormone, enzyme, interferon, factor, or the like. By employing an appropriate regulatory initiation region, inducible production of the deficient protein is achieved so that production of the protein parallels natural production, even though production is in a different cell type from the cell type that normally produces such protein. A ribozyme, antisense or other message can be inserted to inhibit particular gene products or susceptibility to disease.

10 (5) To repair sites of vascular injury. Engineered endothelial stem cells secreting such factors as tissue plasminogen activator, designed to help prevent restenosis after balloon angioplasty, can be infused. Asahara et al (1997) have demonstrated that endothelial stem cells  
15 selectively home to sites of vascular damage.

(6) To isolate new molecules that are important in stem and tumor cell biology.

(7) To form endothelium, stroma and blood cells, depending on the need at the time.

20 The stem cells of the present invention can be expanded in number by long-term *in vitro* culture with minimal differentiation until needed. However, the stem cells can produce blood cells when treated with the appropriate hematopoietic growth and differentiation factors, or form  
25 tubular network structures characteristic of endothelial cells upon treatment with the appropriate agents, or form fibroblast-like stromal cells upon treatment with the appropriate agents. Additionally, the cultured stem cells of the present invention can mature into functionally competent  
30 blood cells *in vivo*, capable of mediating antigen-specific immune responses, repopulating lympho-hematopoietic organs, and prolonging survival of animals with a destroyed hematopoietic system.

Although the phenotype of the particular stem cells  
35 isolated according to the present invention is new, one skilled in the art could readily, without undue

experimentation, be able to use the stem cells of the present invention in the capacities described above, as well as in other capacities. Illustrations of such use are found in Wagner et al, U.S. Patent 5,744,347.

5       The following materials and methods are applicable to all of the following examples.

### Materials and Methods

#### Source of Human Cells

Fresh or frozen cells from bone marrow, cytokine  
10 mobilized peripheral blood or cord blood were obtained from donors after informed consent.

Bone marrow or cord blood samples were diluted with 4 volumes RPMI 1640 medium containing 10% fetal calf serum (FCS). Mononuclear cells were separated on a Histopaque®-1077  
15 density gradient (Sigma Diagnostics, St. Louis, MO) and washed x 2 with PBS-citrate (PBS containing 13.6 mmol/L sodium citrate, 1 mmol/L adenosine and 2 mmol/L theophylline).

Leukapheresis samples were not normally subjected to Histopaque®-1077 density gradient centrifugation but were  
20 washed X 2 with PBS-citrate before proceeding to the filtration step.

PBS-citrate washed cells (from all sources) were filtered through a 40 µm nylon cell strainer (Falcon 2340, Becton-Dickinson, New Jersey). Cells were resuspended in 2 ml  
25 PBS-citrate and overlaid on a 3 ml PBS-citrate/10% bovine serum albumin (BSA) cushion and then centrifuged for 10 minutes at 200 g at room temperature, to remove platelets (Thoma et al, 1994). This step was repeated once or twice.

If necessary DNase was used to digest DNA from cell  
30 debris.

#### Immunomagnetic Separation

Samples were enriched for CD34<sup>+</sup> cells by one of the following methods:

(a) magnetic activated cell sorting (MACS) columns,  
35 using anti-CD34 antibodies coated onto uniform,

supermagnetic, polystyrene beads (Miltenyi Biotec, Auburn, CA). Separation was carried out on a MiniMACS device according to manufacturer's recommendation (Miltenyi Biotec, Auburn, CA).

(b) magnetic separation using the Dynal CD34 Progenitor Cell Selection System (Dynal A.S., Oslo, Norway), which also uses anti-CD34 antibodies immobilized onto microbeads.

10 In both techniques magnetically labeled cells are separated from other cells by means of a magnetic field.

#### Antibodies and Reagents

The following antibodies were used for immunofluorescent staining: CD34-FITC, CD34-PE, CD34-RPE-CY5, c-kit-PE, CD38-FITC, Mouse IgG-FITC, Mouse IgG-PE, Mouse IgG-RPE-Cy5, Mouse IgG2a, streptavidin-PE and goat-anti-mouse-RPE were purchased from Dako (Dako A/S, Glostrup, Denmark). Thy-1-FITC and Thy-1-PE were obtained from Immunotech (Immunotech, France). CD31-FITC, Mouse IgG Biotin, CD34-APC and Mouse IgG APC were obtained from Caltag (Caltag Laboratories, Burlingame, CA). HLA-DR-FITC, Mouse IgG and Goat IgG were purchased from Sigma (Sigma®, St. Louis MO) and CD34-APC, CD31-PE, Mouse IgG-APC from Becton-Dickinson (Becton-Dickinson, San Jose, CA). AC133-PE was obtained from Miltenyi (Miltenyi Biotec, Auburn, CA). VE-Cadherin-FITC was a kind gift from Dr. W. A. Muller, Cornell University, New York. TIE 1-FITC, TIE 1-PE, biotinylated TIE 1 and biotinylated TEK were generous gifts from Dr. T. Suda, Kumamoto University, Kumamoto, Japan. FGF-R1 antibody was obtained from Dr. W. L. McKeehan, Texas A and M University, Houston, TX or commercially, from QED Bioscience Inc. (San Diego, CA). Anti-FGF-R1-FITC was either purchased from QED or prepared in our laboratory. Anti-FGF-R1-APC was produced, purified and conjugated to allophycocyanin (APC) in our own laboratory.

35 Conjugation of the antibody to APC was performed using a Phycolink™ conjugation kit, PJ25C, purchased from Prozyme

(Prozyme, San Leandro, CA).

#### Cell Staining and Flow Cytometry

MACS-selected or Dynal-selected CD34<sup>+</sup> cells were resuspended in PBS/0.1% BSA/0.01% NaN<sub>3</sub>/Aprotinin 20μg/ml (PBS/BSA/N<sub>3</sub>/Aprotinin). Fc receptors and non specific binding of immunoglobulins to cell surfaces were blocked with human IgG and either mouse or goat IgG where appropriate. Cells were incubated with appropriate antibodies for 30 minutes on ice. After washing x 2 with PBS/BSA/N<sub>3</sub>/Aprotinin, cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA), equipped with an argon laser to excite FITC, PE and RPE-CY5 fluorochromes and a helium-neon diode, with time delay adjusted according to manufacturer's recommendations, for excitation of allophycocyanine (APC). 30,000 to 150,000 CD34<sup>+</sup> selected cells were analyzed using CellQuest software (Becton-Dickinson, San Jose, CA). The dye, 7-aminoactinomycin D (7-AAD) (Sigma, St. Louis, MO), was used in some experiments to identify dead cells. This was done to ensure that the CD34<sup>+</sup>FGFR<sup>+</sup> population was a viable population.

#### Cell Sorting

To isolate and study the growth characteristics of the CD34<sup>+</sup>FGFR<sup>+</sup> population, CD34<sup>+</sup> selected cells were obtained as previously described. These cells were incubated with antibodies to CD34 and FGF-R1 (as described above) and sorted on a Coulter Epics Elite (Beckman Coulter Inc., Fullerton, CA) into CD34<sup>+</sup>FGFR<sup>+</sup> and CD34<sup>+</sup> FGFR<sup>-</sup> populations. Sorted cells were incubated in a variety of media, including RPMI 1640, αMEM, DMEM and long term culture medium (LTCM) (Myelocult H5100 from StemCell Technologies Inc., Vancouver, Canada) containing 12.5% horse serum and 12.5% FCS in the presence or absence of growth factors such as FGF-2 and VEGF. Growth was assessed by determining the number of cells present at various time points.

#### EXAMPLE 1: CD34<sup>+</sup>FGFR<sup>+</sup> Co-Expression of Other Antigens

CD34-enriched cells were purified from cytokine



mobilized peripheral blood (PB), bone marrow (BM), or cord blood (CB), using magnetic separation techniques (Dynal or MiniMacs). Fluorescent-labeled antibodies were used to assay for CD34<sup>+</sup>FGFR<sup>+</sup> cells and a percentage of CD34<sup>+</sup>FGFR<sup>+</sup> was  
5 determined for each experiment. The results of the fourteen experiments which were run are shown in Table 1. For the fourteen experiments, a mean of 4.4% ( $\pm$  2.3%) of CD34<sup>+</sup> cells expressed FGFR.

The presence of a third antigen on the CD34<sup>+</sup>FGFR<sup>+</sup>  
10 cells was also ascertained using fluorescent antibodies. The mean percentage of CD34<sup>+</sup>FGFR<sup>+</sup> cells expressing a particular third antigen is shown in Table 1 and is shown graphically in Figure 1. The error bars indicate the standard deviation (SD) and the numbers show the number of experiments assaying a  
15 particular antigen.

The experiments were run by submitting the CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> enriched cells to the Becton-Dickinson FACSCalibur machine using the CellQuest System software for three- and four-color analysis. The techniques were similar to those  
20 described in "Flow Cytometry Analysis Using the Becton-Dickinson FACScan", Current Protocols in Immunology, unit 5.4, pages 5.4.1-5.4.19 (Supplement 16, 1995).

TABLE 1

				% CD34 <sup>+</sup> FGFR <sup>+</sup> Cells Expressing the Indicated Third Antigens								
Experiment #	Source	Selection Method	% CD34 <sup>+</sup> FGFR <sup>+</sup>	CD38	HLA-Dr	AC133	Thy-1	c-kit	CD31	Tie 1	TEK	VE-Cadherin
1	PB	Dyn	3.2	100	43		97					
2	PB	Macs	7.9						66			
3	PB	Macs	3.6				50		38			
4	PB	Macs	3.1	100			38		85	29		
5	PB	Macs	3.9			50						
6	PB	Macs	6.6			67	26	94	99	23		
7	PB	Macs	3.6	96	53	73	57	40	97	47	25	85
8	PB	Macs	8.2			76	87				68	99
9	BM	Dyn	4.9				94	78	52			
10	BM	Macs	2.1				29	28	74			
11	CB	Dyn	5.8									
12	CB	Macs	1.3									
13	CB	Macs	6.5				31					
14	CB	Macs	1.1			55	23	67				
		Mean	4.4	99	48	64	52	57	70	33	47	86
		SD	2.3	2	7	11	28	27	23	12	30	12
		n	14	3	2	5	11	6	7	3	2	3

SD = Standard Deviation  
n = Number

99% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express CD38 (range 96%-100%)  
86% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express VE-Cadherin (range 75%-99%)  
70% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express CD31 (range 38%-99%)  
64% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express AC133 (range 50%-76%)  
57% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express c-kit (range 28%-94%)  
52% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express Thy-1 (range 23%-97%)  
48% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express HLA-Dr (range 43%-68%)  
47% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express TEK (range 25%-68%)  
33% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express Tie-1 (range 23%-47%)

It can be seen that in Experiment 7 results were obtained for third antigens from each of the panel of nine different antigens. The results of Experiment 7 are tabulated in Table 2. Furthermore, flow cytometry plots for Experiment 5 7 are shown in Figures 2A-2F. Figure 2A is a dot plot of forward scatter (FSC) versus side scatter (SSC) of all events. FSC gives an indication of cell size, i.e., low FSC equals small size. SSC gives an indication of cell granularity or complexity, i.e., low SSC equals low granularity. A region R1 10 is drawn to delineate cells of low-to-high FSC and low-to-medium SSC. This eliminates the area containing most of the cell debris and doublets.

Figure 2B is a histogram showing the intensity of staining with the dye 7-AAD (Philpott et al, 1996). 7-AAD is 15 a large molecule which is not readily taken up by cells with intact cell membranes and which stains dead cells intensely. The histogram is gated on R1, and the region R2 is drawn to delineate live cells.

Figure 2C is a dot plot of SSC versus CD34 gated on 20 R1 AND R2. The gates are set using Boolean logic in which the convention used for R1 AND R2 is  $R1 \cdot R2$ , and in this instance the gate delineates live cells in R1. The region R3 is drawn to delineate CD34<sup>+</sup> cells in this plot. FITC was used to label the CD34<sup>+</sup> cells.

25 Figure 2D is a dot plot of FSC versus SSC gated on R1 AND R2 AND R3 ( $R1 \cdot R2 \cdot R3$ ), i.e., this plot uses "backgating" to show the FSC/SSC characteristics of live CD34<sup>+</sup> cells in R1.

Figure 2E is a dot plot of CD34 versus FGFR gated on R1 AND R2, i.e., gated on live cells in R1. The region R4 is 30 drawn to delineate CD34<sup>+</sup>FGFR<sup>+</sup> cells.

Figure 2F is a dot plot of FSC versus SSC gated on R1 AND R2 AND R4 ( $R1 \cdot R2 \cdot R4$ ), i.e., this plot uses "backgating" to show the characteristics of live CD34<sup>+</sup>FGFR<sup>+</sup> cells in R1. It is noted that the majority of live CD34<sup>+</sup>FGFR<sup>+</sup> cells have low 35 FSC and low SSC, i.e., they are small cells with low granularity. The position of these cells on the scatter dot

plot is somewhat unusual, as it appears in the region which is conventionally ignored.

TABLE 2  
Experiment 7:  
Analysis of Live CD34<sup>+</sup>FGFR<sup>+</sup> Population from Cytokine Mobilized Peripheral Blood

Number of CD34 <sup>+</sup> Cells	Number of CD34 <sup>+</sup> FGFR <sup>+</sup> Cells	% CD34 <sup>+</sup> FGFR <sup>+</sup> (% of CD34 <sup>+</sup> )	Phenotype	Number of Cells of Each Phenotype	Phenotype (% of CD34 <sup>+</sup> Population)	Phenotype (% of CD34 <sup>+</sup> FGFR <sup>+</sup> Population)
90121	4039	4.48	CD34 <sup>+</sup> FGFR <sup>+</sup> CD38 <sup>+</sup>	3876	4.30	96
104819	4829	4.61	CD34 <sup>+</sup> FGFR <sup>+</sup> HLA-DR <sup>+</sup>	2561	2.44	53
90803	2910	3.20	CD34 <sup>+</sup> FGFR <sup>+</sup> AC133 <sup>+</sup>	2106	2.32	73
111007	4773	4.30	CD34 <sup>+</sup> FGFR <sup>+</sup> c-kit <sup>+</sup>	1923	1.73	40
100291	2805	2.80	CD34 <sup>+</sup> FGFR <sup>+</sup> THY-1 <sup>+</sup>	1594	1.59	57
86441	2957	3.42	CD34 <sup>+</sup> FGFR <sup>+</sup> CD31 <sup>+</sup>	2869	3.32	97
8478	2956	3.50	CD34 <sup>+</sup> FGFR <sup>+</sup> VE-Cadherin <sup>+</sup>	2524	2.99	85
100148	3969	3.96	CD34 <sup>+</sup> FGFR <sup>+</sup> TIE <sup>+</sup>	1866	1.86	47
100758	2491	2.47	CD34 <sup>+</sup> FGFR <sup>+</sup> TEK <sup>+</sup>	686	0.68	28

Mean: 3.64  
SD 0.75

Mean % CD34<sup>+</sup> cells per sample = 98% ( $\pm 0.65$ )

Table 3 shows the results of some of the samples from Experiment 8. In these samples, CD34 staining was not included in order that other combinations of antigens could be assayed. Figure 3 shows these results graphically. One can see that all live FGFR<sup>+</sup>Thy-1<sup>+</sup> cells co-express VE-Cadherin as do 97% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells. Since 99% of CD34<sup>+</sup>FGFR<sup>+</sup> cells in Experiment 8 were found to be VE-Cadherin<sup>+</sup>, one can extrapolate from this experiment that the phenotype of the CD34<sup>+</sup>FGFR<sup>+</sup> population is CD34<sup>+</sup>FGFR<sup>+</sup>VE-Cadherin<sup>+</sup>Thy-1<sup>+</sup>AC133<sup>+</sup> and that approximately two-thirds are TEK<sup>+</sup>.

Table 3  
Experiment 8: Analysis of Live FGFR<sup>+</sup> Cells from  
Cytokine Mobilized Peripheral Blood

Sample #	Number of LIVE FGFR <sup>+</sup> Cells		Number of Cells of Each Phenotype	% of LIVE FGFR <sup>+</sup> Population	
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup>	609	96	
1	635	LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup>	534	84	1a
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup> THY-1 <sup>+</sup>	535	84	1b
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup>	550	93	
2	594	LIVE FGFR <sup>+</sup> AC133 <sup>+</sup>	316	53	2a
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup> AC133 <sup>+</sup>	307	52	2b
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup>	270	76	
3	356	LIVE FGFR <sup>+</sup> AC133 <sup>+</sup>	198	56	3a
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup> AC133 <sup>+</sup>	181	51	3b
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup>	441	70	
4	627	LIVE FGFR <sup>+</sup> TEK <sup>+</sup>	399	64	4a
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup> TEK <sup>+</sup>	269	43	4b

Table 3 shows that in this experiment:

- All live FGFR<sup>+</sup>THY-1<sup>+</sup> cells co-express VE-Cadherin (1a, 1b)
- About 97% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express VE-Cadherin (2a, 2b)
- At least 91% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express THY-1 (3a, 3b)
- At least 67% of live FGFR<sup>+</sup>TEK<sup>+</sup> cells co-express THY-1 (4a, 4b)

#### Example 2: CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> Co-Expression of Other Antigens

The following experiment was conducted using the techniques described for Example 1. Lineage depletion to obtain lin<sup>-</sup> cells was conducted as described in Bhatia et al, 1997, and Bhatia et al, 1998, using Dynal beads and magnetic separation. The results obtained are summarized in Table 4.

It can be seen that 77% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express CD31, 40% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express c-kit, 47% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express VE-Cadherin, and 18% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express TIE-1.

5

TABLE 4

Expt	CD34	% of CD34 <sup>+</sup> or CD34-LIN <sup>-</sup> FGFR <sup>+</sup> Cells Expressing Third Antigen	Cell Number
(a)	+	96% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are CD31 <sup>+</sup>	572
	-	77% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are CD31 <sup>+</sup>	211
	+	97% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are c-kit <sup>+</sup>	1500
	-	40% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are c-kit <sup>+</sup>	107
(b)	+	75% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are VE-Cadherin <sup>+</sup>	417
	-	47% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are VE-Cadherin <sup>+</sup>	570
	+	42% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	237
	-	13% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	162
(c)	+	37% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	1133
	-	23% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	1625

#### 10 Example 3: Growth Characteristics

Cytokine mobilized peripheral blood cells were enriched for CD34<sup>+</sup> cells using magnetic separation. The cells were stained with fluorescent-labeled antibodies to CD34 and FGFR and sorted on a fluorescence-activated cell sorter (FACS) into FGFR<sup>+</sup> and FGFR<sup>-</sup> populations. Cells were seeded at 1000 cells per well into collagen/gelatin coated wells containing long-term culture medium (LTCM) with either (a) no additions, (b) 10 ng/ml FGF-2 or (c) 10 ng/ml FGF-2 plus 10 ng/ml VEGF, and incubated at 37°C in a humidified incubator. After 4-6 weeks, the number of cells per well were counted. The results are shown in Table 5 and shown graphically in Figure 4. It can be seen that the FGFR<sup>+</sup> population grows in an FGF dependent manner and that the addition of VEGF together with FGF-2

further increases the growth of the cells.

**Table 5**  
**Cell Growth**  
(cells/well)

5

Expt	Population	ADDITIONS		
		None	FGF-2 (10 ng/ml)	FGF-2 + VEGF (both at 10 ng/ml)
(i)	CD34 <sup>+</sup> FGFR <sup>+</sup>	1734	11978	ND
	CD34 <sup>+</sup> FGFR <sup>-</sup>	1244	2500	ND
(ii)	CD34 <sup>+</sup>	1617	1457	2311
	CD34 <sup>+</sup> FGFR <sup>+</sup>	3227	8057	17140
	CD34 <sup>+</sup> FGFR <sup>-</sup>	1493	2098	3093

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 15 the references cited within the references cited herein are  
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Reference to known method steps, conventional method  
 steps, known methods or conventional methods is not in any way  
 an admission that any aspect, description or embodiment of the  
 20 present invention is disclosed, taught or suggested in the  
 relevant art.

The foregoing description of the specific  
 embodiments will so fully reveal the general nature of the  
 invention that others can, by applying current knowledge,  
 25 readily modify and/or adapt for various applications such  
 specific embodiments without undue experimentation and without  
 departing from the generic concept, and, therefore, such  
 adaptations and modifications should and are intended to be  
 comprehended within the meaning and range of equivalents of  
 30 the disclosed embodiments. It is to be understood that the  
 phraseology or terminology employed herein is for the purpose



of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means  
5 for...", or any method step language, as may be found in the specification above and/or in the claims below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the  
10 future exist which carries out the recited function, whether or not precisely equivalent to the embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same function can be used; and it is intended that such expressions be given their broadest  
15 interpretation.

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enriched by flow cytometry", Blood 90:3471-3481 (1997).

## WHAT IS CLAIMED IS:

1. A composition comprising a physiologically acceptable medium and human stem cells, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and also having another indicator of a primitive state.

2. A composition in accordance with claim 1, wherein said indicator of a primitive state is selected from the group consisting of CD34<sup>+</sup>, CD34<sup>-</sup>lin<sup>-</sup>, being an embryonic stem cell, Thy-1<sup>+</sup>, AC133<sup>+</sup> and c-kit<sup>+</sup>.

3. A composition in accordance with claim 2, wherein said indicator of a primitive state is CD34<sup>+</sup>.

4. A composition in accordance with claim 2, wherein said indicator of a primitive state is CD34<sup>-</sup>lin<sup>-</sup>.

5. A composition in accordance with claim 2, wherein said indicator of a primitive state is the state of being an embryonic stem cell.

6. The composition according to claim 1, wherein said human stem cells are selected from the group consisting of endothelial stem cells, stromal stem cells and hematopoietic stem cells.

7. The composition according to claim 1, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more of the markers Thy-1<sup>+</sup>, AC133<sup>+</sup> and c-kit<sup>+</sup>.

8. The composition according to claim 1, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

9. A composition in accordance with claim 8, wherein said markers indicative of endothelial cells are selected from the group consisting of TIE-1<sup>+</sup>, TEK<sup>+</sup>, CD31<sup>+</sup>, VE-Cadherin<sup>+</sup> and VEGFR<sup>+</sup>.

10. The composition according to claim 1, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further

having one or more markers indicative of stromal cells.

11. A composition in accordance with claim 10, wherein said marker indicative of stromal cells is STRO-1<sup>+</sup>.

12. The composition according to claim 7, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

13. The composition according to claim 7, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

14. A composition in accordance with claim 1, wherein said human stem cells are a subpopulation of peripheral blood cells, bone marrow cells, or cord blood cells.

15. A composition in accordance with claim 1, wherein said human stem cells are a subpopulation of embryonic stem cells.

16. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and also having another phenotype indicative of a primitive state.

17. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more of the markers Thy-1<sup>+</sup>, AC133<sup>+</sup> and c-kit<sup>+</sup>.

18. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

19. A composition in accordance with claim 18, wherein said markers indicative of endothelial cells are selected from the group consisting of TIE-1<sup>+</sup>, TEK<sup>+</sup>, CD31<sup>+</sup>, VE-Cadherin<sup>+</sup> and VEGFR<sup>+</sup>.

20. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells

characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

21. A composition in accordance with claim 20, wherein said marker indicative of stromal cells is STRO-1<sup>+</sup>

22. The composition according to claim 7, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

23. The composition according to claim 7, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

24. A composition in accordance with claim 16, wherein said human stem cells are a subpopulation of peripheral blood cells, bone marrow cells, or cord blood cells.

25. A composition in accordance with claim 16, wherein said human stem cells are a subpopulation of embryonic stem cells.

26. A cellular composition comprising a substantially homogeneous population of cultured human stem cells displaying a phenotype of FGFR<sup>+</sup> and another phenotype indicative of a primitive state, which cells are capable of giving rise to cells selected from the group consisting of endothelial cells, stromal cells and hematopoietic cells.

27. A suspension of human cells comprising pluripotent stem cells substantially free of mature lymphoid and myeloid cells.

28. The suspension according to claim 27, wherein said stem cells are selected from the group consisting of endothelial cells, stromal cells and hematopoietic cells.



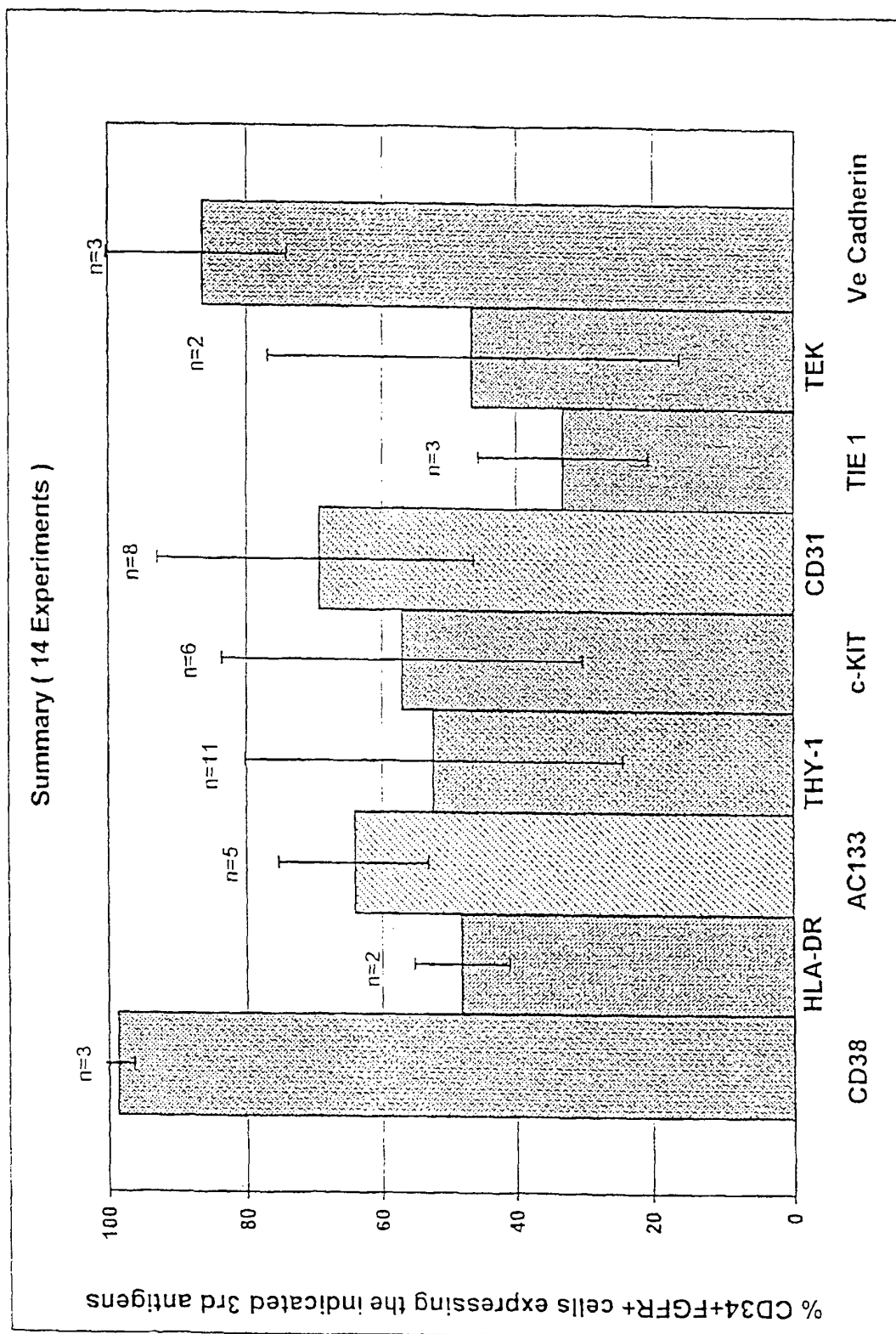


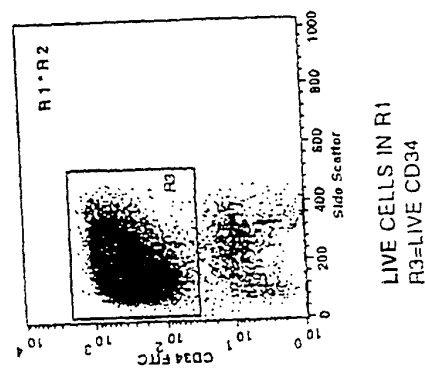
FIGURE 1

# EXPERIMENT 7

WO 00/12683

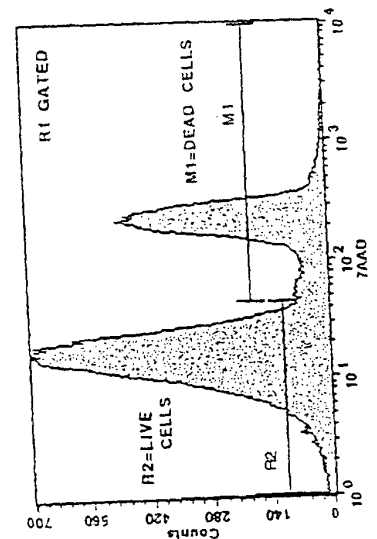
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LIVE CELLS IN R1  
R3=LIVE CD34

FIGURE 2C



R2 GATES THE 7AAD NEGATIVE CELLS  
I.e. R2=LIVE CELLS

FIGURE 2B

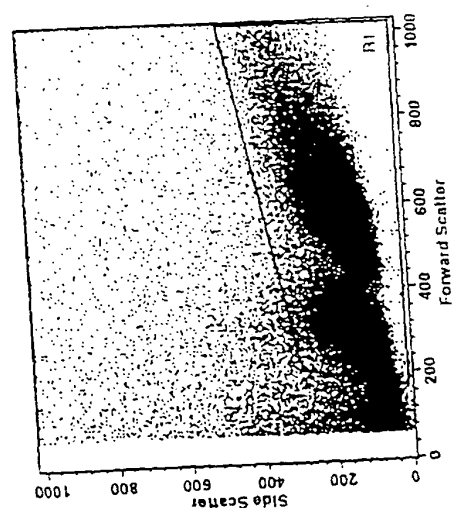
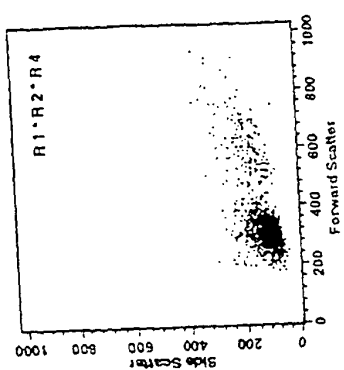
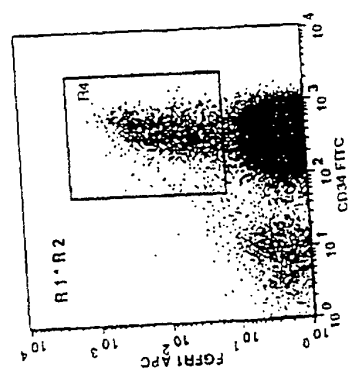


FIGURE 2A



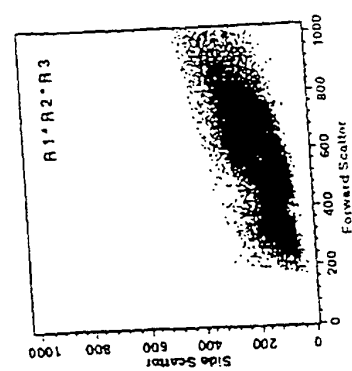
LIVE CD34+FGFR+ CELLS IN R1

FIGURE 2F



LIVE CELLS IN R1  
R4=LIVE CD34+FGFR+

FIGURE 2E



LIVE CD34+ CELLS IN R1

FIGURE 2D

Experiment 8

( Analysis of live FGFR+ population )

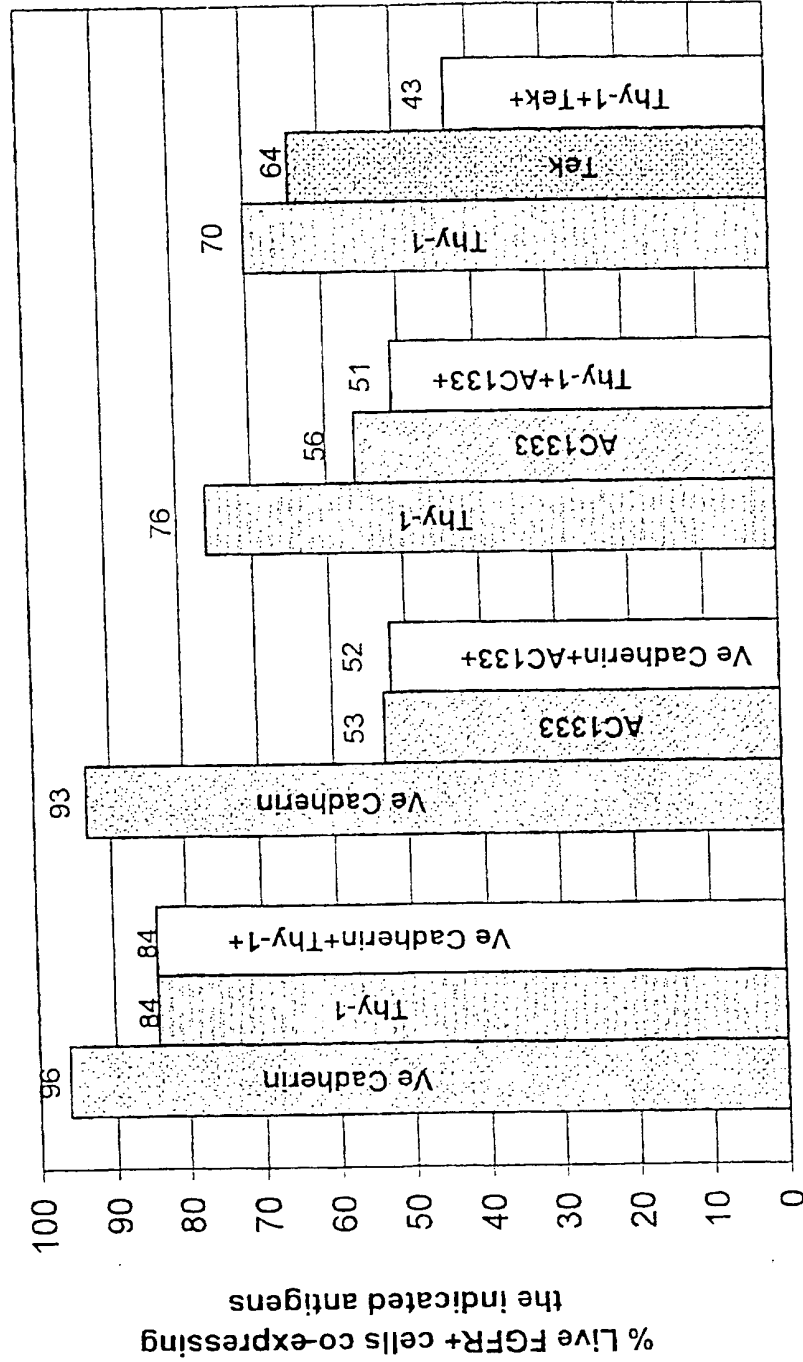


FIGURE 3

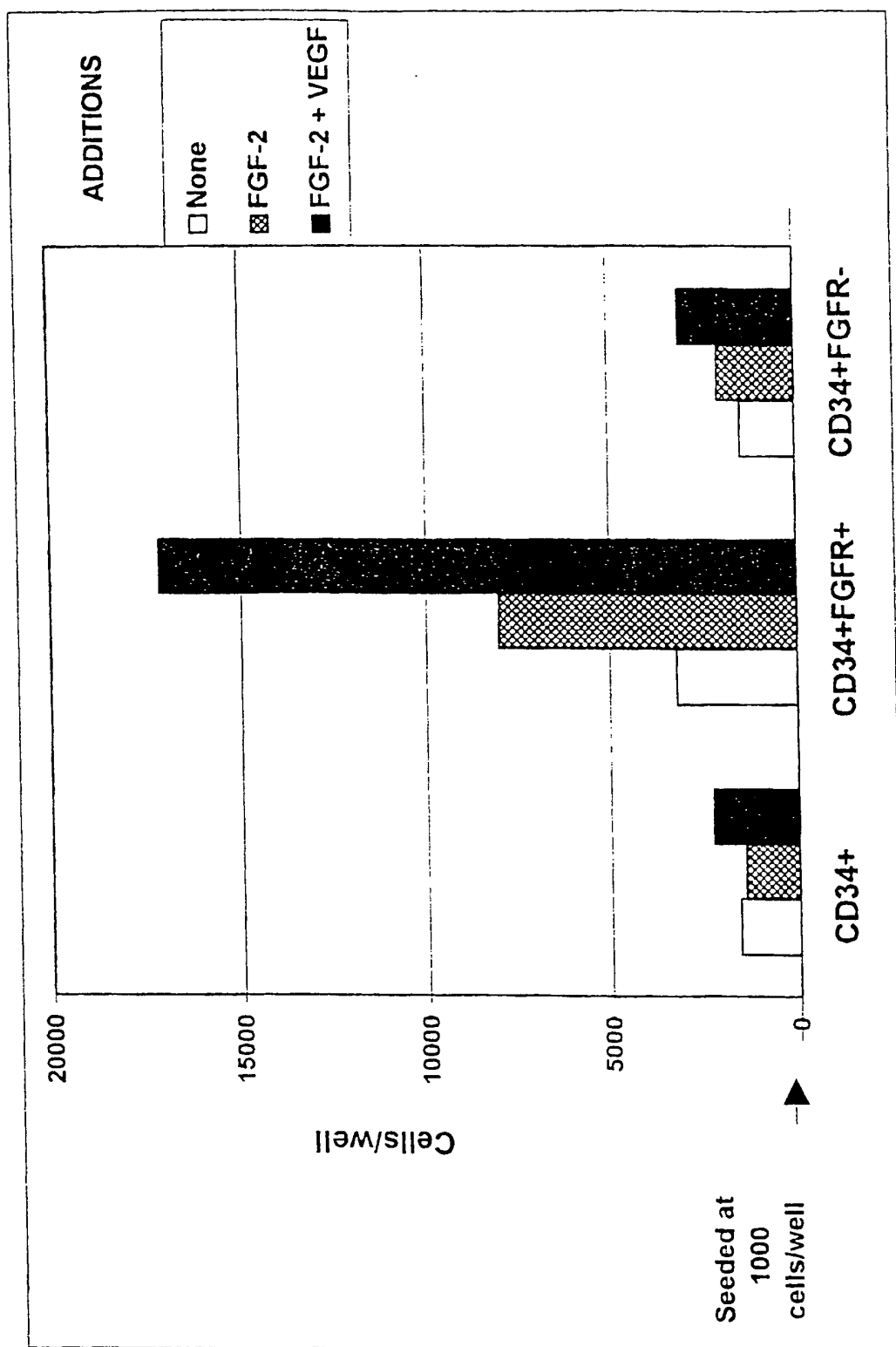


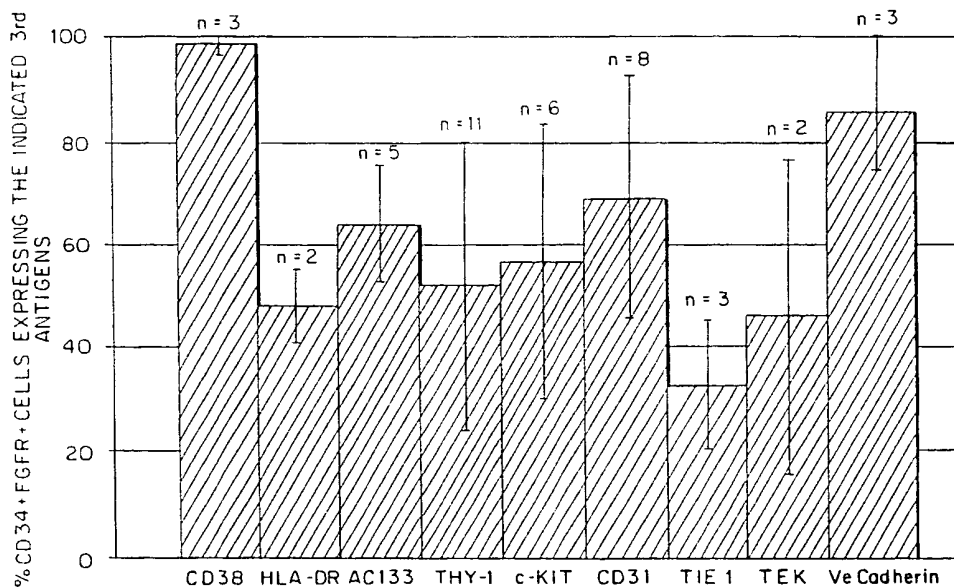
FIGURE 4



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>7</sup> : <b>C12N 5/08, A61K 35/14, 35/28, A61P 43/00</b>		<b>A3</b>	(11) International Publication Number: <b>WO 00/12683</b>
			(43) International Publication Date: 9 March 2000 (09.03.00)
(21) International Application Number: PCT/US99/19730			(81) Designated States: AU, CA, IL, JP, ZA, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).
(22) International Filing Date: 31 August 1999 (31.08.99)			
(30) Priority Data: 60/098,480 31 August 1998 (31.08.98) US			
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Published With international search report.			

(54) Title: STEM CELLS BEARING AN FGF RECEPTOR ON THE CELL SURFACE



## (57) Abstract

A composition of substantially purified pluripotent stem cells are positive both for fibroblast growth factor receptor (FGFR) and a phenotype indicative of a primitive state, such as CD34<sup>+</sup>, CD34<sup>+</sup>lin<sup>-</sup>, Thy-1<sup>+</sup>, AC133<sup>+</sup> or c-kit<sup>+</sup>. The state of being an embryonic stem cell is also a phenotype indicative of a primitive state. This population may be further defined by the subpopulations thereof which have another marker thereon indicative of endothelial cells, such as TIE-1<sup>+</sup>, TEK<sup>+</sup>, CD31<sup>+</sup>, VE-Cadherin<sup>+</sup> or VEGFR<sup>+</sup> or indicative of stromal cells, such as STRO-1<sup>+</sup>.

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**STEM CELLS BEARING AN FGF RECEPTOR  
ON THE CELL SURFACE**

**FIELD OF INVENTION**

5           The present invention is directed to a new  
phenotype of stem cells which contain a fibroblast growth  
factor receptor (FGFR) on the cell surface thereof and  
further have a phenotype indicative of a primitive state.  
The present invention is further directed to subpopulations  
10 thereof having a phenotype indicative of endothelial or  
stromal cells.

**BACKGROUND OF THE INVENTION**

          The ability of tissues and organs to develop,  
remodel, regenerate, and repair depends on the existence of  
15 stem cells (also known as progenitor cells) that, upon  
division, form more differentiated progeny. Stem cells have  
been found in the epidermis, the intestinal epithelium, and  
the hematopoietic system. There is mostly indirect evidence  
of stem cells in mesenchymal tissues. *In vivo* and *in vitro*  
20 studies have provided evidence of osteogenic precursor cells  
in bone marrow and other stromal cell preparations. However,  
the identity of cells in these tissues and their relationship  
to cells with classical stem cell characteristics have yet to  
be established.

25           Endothelial cells are part of the normal bone  
marrow stroma. Long-term cultures of human bone marrow  
contain a complex mix of stromal cells including adipocytes,  
fibroblasts, endothelial cells, macrophages, and smooth  
muscle cells. Endothelial cells and hematopoietic cells are  
30 thought to be derived from the common progenitor cells,  
hemangioblasts.

          Cell surface molecules on various types of cells,  
and particularly hematopoietic cells, are given a cluster of  
differentiation (CD) designation in which each CD molecule  
35 designation describes a surface molecule (marker)  
identifiable by a cluster of monoclonal antibodies that

display the same cellular reactivity. CD designations are assigned at regularly held international workshops on human leukocyte differentiation antigens. For example, the CD19 marker is specific to B cells, and the CD33 marker is  
5 specific to myeloid cells. At the present time, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker which has been indicated as being present on stem cells is CD34. However, this marker is also found on a significant number of  
10 lineage-committed progenitors. Other markers which are known or thought to be present primarily on stem cells, i.e., "primitive" markers, include AC133 (Yin et al, 1997; Buhring et al, 1999), Thy-1 (Murray et al, 1995) and c-kit (D'Arena et al, 1998).

15 It is known that a small number of circulating CD34<sup>+</sup> hematopoietic stem cells are present in peripheral blood. As the major source of CD34<sup>+</sup> hematopoietic stem cells in the adult is the bone marrow, the purpose of this small, circulating CD34<sup>+</sup> cell population is unknown. One explanation  
20 is that the bone marrow is "leaky", and the stem cells escape, circulate and return to the marrow. A second possibility is that the function of these circulating stem cells is to seed sites, such as the liver and the spleen, which can function as additional sites of hematopoiesis in a  
25 crisis.

The human CD34<sup>+</sup> hematopoietic population isolated from bone marrow, cord blood, and peripheral blood is a heterogeneous population that contains hematopoietic stem cells. Recent evidence indicates that circulating CD34<sup>+</sup> cells  
30 also contain endothelial stem cells, which may also circulate (Asahara et al, 1997; Nieda et al, 1997; Shi et al, 1998; Lin et al, 1998). Asahara et al (1997) have shown that CD34<sup>+</sup> cells isolated from the peripheral blood can be incorporated



into the endothelium of ischaemic blood vessels of recipient animals. Purified umbilical cord blood CD34<sup>+</sup> cells also give rise to von Willebrand factor-expressing endothelial cells *in vitro*, providing additional evidence for a circulating  
5 progenitor endothelial cell (Nieda et al, 1997). In addition, bone marrow derived CD34<sup>+</sup> cells also contain a transplantable stromal stem cell (Prockop, 1997; Pereira et al, 1998).

Recently, convincing evidence has been presented  
10 (Goan et al, 1997) that human CD34<sup>+</sup> progenitor cells from peripheral blood or cord blood that were transplanted into NOD/SCID immunodeficient mice gave rise to human stromal cells. The human stromal cells expressed the endothelial cell-specific vascular endothelial growth factor (VEGF)  
15 receptor-2 (KDR) and von Willebrand factor, indicating that they were of endothelial origin. There is also recent evidence that infusion of whole bone marrow cells into recipient mice results in fibroblasts of donor origin in a number of non-hematopoietic tissues (Prockop, 1997; Pereira,  
20 1998), indicating that stromal progenitor cells reside in the bone marrow. As CD34 has been shown to be expressed by bone marrow stromal precursor cells (Simmons et al, 1991), it is possible that these stromal progenitors reside in the bone marrow within the CD34<sup>+</sup> progenitor population.

25 The CD34<sup>+</sup> progenitor population is, therefore, a heterogeneous fraction that may include precursor cells of the hematopoietic, endothelial, and stromal/fibroblast lineages. In addition, pluripotent mesenchymal stem cells capable of differentiating into cells of the osteogenic,  
30 chondrogenic, tendonogenic, adipogenic and myogenic lineages have been shown to reside within the bone marrow microenvironment (Majumdar et al, 1998). There is recent literature indicating that circulating endothelial

progenitor/stem cells exist, and that stromal stem cells in marrow serve as a source for continual renewal of cells in a number of non-hematopoietic tissues. A common embryological precursor that gives rise to both hematopoietic and  
5 endothelial cells has recently been identified (Suda et al, 1997; Choi et al, 1998; Caprioli et al, 1998).

Recent evidence has also shown that embryonic stem (ES) cells can give rise to endothelial cells (Hirashima et al, 1999).

10 Fibroblast growth factors (FGFs) can synergize with other factors to stimulate hematopoietic progenitor cell proliferation (Wilson et al, 1991; Quito et al, 1996; Allouche, 1995; Yuen et al, 1998, U.S. Patent 5,612,211; U.S. Patent 5,817,773). It has also been shown that basic FGF  
15 (FGF-2) acts to antagonize cytokines that induce differentiation (Burger et al, 1994). In addition, low amounts of FGF-2, on the order of 10-100 pg/ml, induce a more primitive phenotype in human K562 leukemic cells.

It would be very useful to be able to isolate stem  
20 cells which are progenitors of endothelial and/or stromal cells. The more primitive the stem cell, the more useful it is in bone marrow transplantation. Furthermore, endothelial stem cells and stromal stem cells, or a stem cell which is a progenitor of both, would find many utilities in repairing  
25 damaged vasculature and in treating other conditions where endothelial or stromal cells need to be replenished.

#### **SUMMARY OF THE INVENTION**

It is an object of the present invention to overcome the deficiencies in the prior art.

30 It is another object of the present invention to isolate a new phenotype of stem cells.

It is a further object of the present invention to identify and isolate stem cells for use in stem cell

transplantation.

It is yet another object of the present invention to identify and isolate endothelial and/or stromal stem cells.

5           A small population of cells having a "primitive phenotype", such as CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup>, has been isolated which express cell surface receptors for fibroblast growth factor (FGF). The population of cells bearing FGF receptors (FGFR) are designated as FGFR<sup>+</sup>. The FGFR<sup>+</sup> primitive phenotype  
10 cell population has several unique properties:

(1) The CD34<sup>+</sup>FGFR<sup>+</sup> cells are predominantly present in the region of the fluorescence-activated cell sorter profile having low forward scatter (FSC) and low side scatter (SSC). Thus, the majority of the cells of the population are  
15 very small and of low granularity. These small cells are located in the FSC/SSC region of the fluorescence-activated cell sorter profile that is normally not analyzed, as this area contains many of the dead and apoptotic cells. Interestingly, this region has recently been shown to be the  
20 site of a mesenchymal stem cell population (Zohar et al, 1997). The CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells have FSC/SSC properties that are similar to those of the CD34<sup>+</sup>FGFR<sup>+</sup> cells. This CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup> population also includes significant numbers of cells with higher FSC properties.

25           (2) The CD34<sup>+</sup>FGFR<sup>+</sup> cells are deeply dormant, which is characteristic of a stem cell population. They do not proliferate in culture until 30-60 days after isolation.

The FGFR<sup>+</sup> primitive phenotype cell population is a unique stem cell population that is a precursor cell for  
30 endothelium and/or hematopoiesis and/or stroma. The FGFR<sup>+</sup> primitive phenotype cell population, obtained either from general circulation, the bone marrow, cord blood or embryonic cells, is capable of forming endothelial, blood and stromal

cells, depending on the need at the time.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a bar graph summarizing the fourteen experiments of Example 1 and showing the percent of CD34<sup>+</sup>FGFR<sup>+</sup> cells which express the indicated third antigens.

Figures 2A-2F are flow cytometry plots for Experiment 7 of Example 1. Figure 2A is a dot plot of FSC versus SSC of all events with region R1 drawn to eliminate the area containing most of the cell debris and doublets. Figure 2B is a histogram showing the intensity of staining with the dye 7-aminoactinomycin D (7-AAD). The region R2 is drawn to delineate live cells. Figure 2C is a dot plot of SSC versus CD34 gated on R1 AND R2. The region R3 is drawn to delineate CD34<sup>+</sup> cells. Figure 2D is a dot plot of FSC versus SSC gated on R1 AND R2 AND R3, thus showing the FSC/SSC characteristics of live CD34<sup>+</sup> cells in R1. Figure 2E is a dot plot of CD34 versus FGFR gated on R1 AND R2. The region R4 is drawn to delineate CD34<sup>+</sup>FGFR<sup>+</sup>. Figure 2F is a dot plot of FSC versus SSC gated on R1 AND R2 AND R4, thereby showing the characteristics of live CD34<sup>+</sup>FGFR<sup>+</sup> cells in R1.

Figure 3 is a bar graph showing the percent of live FGFR<sup>+</sup> cells co-expressing the indicated antigens.

Figure 4 is a bar graph showing the growth of various cell populations in the presence or absence of FGF-2 or a combination of FGF-2 plus VEGF.

#### **DETAILED DESCRIPTION OF THE INVENTION**

Stem cells, when transplanted, can restore the production of hematopoietic, endothelial and stromal cells to a patient who has lost such production due to, for example, radiation therapy. By isolating FGFR<sup>+</sup> primitive phenotype cells, preferably CD34<sup>+</sup>FGFR<sup>+</sup> or CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup>, from other cells in the body, it is possible to obtain relatively pure stem cells, preferably separate from contaminating cells and

other substances, so that the stem cells can be safely transplanted into a patient in need thereof.

The unique isolated cells of the present invention are separated from other cells by virtue of their CD34<sup>+</sup> or  
5 CD34<sup>-</sup>lin<sup>-</sup> state and possession of fibroblast growth factor receptors. The cells can be isolated by conventional techniques for separating cells, such as those described in Civin, U.S. Patents 4,714,680, 4,965,204, 5,035,994, and 5,130,144, Tsukamoto et al 5,750,397, and Loken et al, U.S.  
10 Patent 5,137,809, all of which are hereby incorporated by reference in their entirety. Thus, for example, a CD34-specific monoclonal antibody or an FGFR-specific antibody can be immobilized, such as on a column or on magnetic beads. The entire cell population may then be passed through the  
15 column or added to the magnetic beads. Those which remain attached to the column or are attached to the magnetic beads, which may then be separated magnetically, are those cells which contain a marker which is recognized by the antibody used. Thus, if the anti-CD34 antibody is used, then the  
20 resulting population will be greatly enriched in CD34<sup>+</sup> cells. If the antibody used is FGFR, then the resulting population will be greatly enriched in FGFR<sup>+</sup> cells. That population may then be enriched in the other marker by repeating the steps using a solid phase having attached thereto an antibody to  
25 the other marker.

Another way to sort CD34<sup>+</sup>FGFR<sup>+</sup> cells is by means of flow cytometry, most preferably by means of a fluorescence-activated cell sorter (FACS), such as those manufactured by Becton-Dickinson under the names FACScan or FACSCalibur. By  
30 means of this technique, the cells having a CD34 marker thereon are tagged with a particular fluorescent dye by means of an anti-CD34 antibody which has been conjugated to such a dye. Similarly, the FGFR marker of the cells are tagged with

a different fluorescent dye by means of an anti-FGFR antibody which is conjugated to the other dye. When the stained cells are placed on the instrument, a stream of cells is directed through an argon laser beam that excites the fluorochrome to  
5 emit light. This emitted light is detected by a photo-multiplier tube (PMT) specific for the emission wavelength of the fluorochrome by virtue of a set of optical filters. The signal detected by the PMT is amplified in its own channel and displayed by a computer in a variety of different  
10 forms—e.g., a histogram, dot display, or contour display. Thus, fluorescent cells which emit at one wavelength, express a molecule that is reactive with the specific fluorochrome-labeled reagent, whereas non-fluorescent cells or fluorescent cells which emit at a different wavelength do not express  
15 this molecule but may express the molecule which is reactive with the fluorochrome-labeled reagent which fluoresces at the other wavelength. The flow cytometer is also semi-quantitative in that it displays the amount of fluorescence (fluorescence intensity) expressed by the cell. This  
20 correlates, in a relative sense, to the number of the molecules expressed by the cell.

Flow cytometers are also equipped to measure non-fluorescent parameters, such as cell volume or light scattered by the cell as it passes through the laser beam.  
25 Cell volume is usually a direct measurement. The light scatter PMTs detect light scattered by the cell either in a forward angle (forward scatter; FSC) or at a right angle (side scatter; SSC). FSC is usually an index of size, whereas SSC is an index of cellular complexity, although both  
30 parameters can be influenced by other factors.

Preferably, the flow cytometer is equipped with more than one PMT emission detector. The additional PMTs may detect other emission wavelengths, allowing simultaneous

detection of more than one fluorochrome, each in individual separate channels. Computers allow the analysis of each channel or the correlation of each parameter with another. Fluorochromes which are typically used with FACS machines  
5 include fluorescein isothiocyanate (FITC), which has an emission peak at 525 nm (green), R-phycoerythrin (PE) , which has an emission peak at 575 nm (orange-red), propidium iodide (PI), which has an emission peak at 620 nm (red), 7-aminoactinomycin D (7-AAD), which has an emission peak at 660  
10 nm (red), R-phycoerythrin Cy5 (RPE-Cy5), which has an emission peak at 670 nm (red), and allophycocyanin (APC), which has an emission peak at 655-750 nm (deep red).

These and other types of FACS machines may have the additional capability to physically separate the various  
15 fractions by deflecting the cells of different properties into different containers.

Any other method for isolating the CD34<sup>+</sup>FGFR<sup>+</sup> population of a starting material, such as bone marrow, peripheral blood or cord blood, may also be used in  
20 accordance with the present invention. The various subpopulations of the present invention may be isolated in similar manners.

The isolated cell population of this invention can be used in therapeutic methods, such as stem cell  
25 transplantation, as well as other therapeutic methods as described below, as well as others that are readily apparent to those skilled in the art. For example, the isolated cell populations can be administered directly by intravenous route to a mammalian patient requiring a bone marrow transplant in  
30 an amount sufficient to reconstitute the patient's hematopoietic and immune system. Precise, effective quantities can be readily determined by those skilled in the art and will depend, of course, upon the exact condition

being treated by the therapy. In many applications, however, an amount containing approximately the same number of stem cells found in one-half to one liter of aspirated marrow should be adequate.

5           Thus, a suspension of human cells from marrow or blood comprising cells which are positive both for CD34 and for fibroblast growth factor receptors, preferably substantially free of cells that are not positive for both CD34 and fibroblast growth factor receptors, can restore the  
10 production of hematopoietic cells to a human lacking production of these cells. A suspension of these isolated cells is administered to a patient in need thereof in an effective amount to restore production of hematopoietic/endothelial/stromal cells.

15           The patients in need of this product are those with a specific requirement for hematopoietic, endothelial or stromal cells. For example, patients with vascular injury, persons with genetic defects in their hematopoietic, stromal or endothelial cells, such as collagen deficiency, adenosine  
20 deaminase deficiency, or clotting factor deficiency. It is expected that the circulating stem cells will selectively home to sites of hematopoietic, endothelial or stromal cell damage/deficiency.

          Other "primitive" phenotype indicators besides  
25 CD34<sup>+</sup> are also known. These include AC133<sup>+</sup> (Buhring et al, 1994; Yin et al, 1997), Thy-1 (Murray et al, 1995) and c-kit<sup>+</sup> (Buhring et al, 1994; D'Arena et al, 1998). Another population of primitive cells is CD34<sup>-</sup>lin<sup>-</sup>. The present inventors have discovered that the CD34<sup>+</sup>FGFR<sup>+</sup> population of  
30 the preferred embodiment of the present invention contains significant amounts of the AC133 marker (approximately 64%), the Thy-1 marker (approximately 52%) or the c-kit marker (approximately 57%). Many of these cells have more than one



primitive marker.

Furthermore, certain markers are known to be endothelial markers. These include VE-Cadherin (also known as CD144) (Vittet et al, 1996), TIE-1 (also known as TIE) (Suda et al, 1997), TEK (also known as TIE-2) (Suda et al, 1997; Hamaguchi et al, 1999) and CD31 (also known as PECAM) (Watt et al, 1993). Significant quantities of each of these markers were also found on the CD34<sup>+</sup>FGFR<sup>+</sup> population. About 86% of this population co-expresses VE-Cadherin, about 70% co-expresses CD31; about 47% co-expresses TEK; and about 33% co-expresses TIE-1. This data indicates that the CD34<sup>+</sup>FGFR<sup>+</sup> population includes a primitive population of cells which are precursors of endothelial cells. The subpopulation with these endothelial markers can be isolated and are also part of the present invention. Certain additional markers are known to be stromal cell markers, such as STRO-1 (Gronthos et al, 1994). A subpopulation of the CD34<sup>+</sup>FGFR<sup>+</sup> cells of the present invention which is also STRO-1<sup>+</sup> is a primitive population of cells which are precursors of stromal cells. The subpopulation with these stromal markers can be isolated and are also part of the present invention.

The results as to co-expression of additional markers were obtained from fluorescence-activated cell sorter (FACS) analysis using specific antibodies to cell surface antigens. The antibodies were labeled with three or four different fluorochromes. The results show that 100% of live FGFR<sup>+</sup>Thy-1<sup>+</sup> cells co-express VE-Cadherin, 97% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express VE-Cadherin, 91% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express Thy-1, and 67% of live FGFR<sup>+</sup>TEK<sup>+</sup> cells co-express Thy-1. Cell sorter systems using additional fluorochromes will be able to allow the separation of those cells in the CD34<sup>+</sup>FGFR<sup>+</sup> population which also express two or more of the various other primitive or endothelial markers

discussed above. Similarly, other markers, such as the vascular endothelial growth factor-receptor (VEGF-R) (also known as KDR), which is a marker for endothelial cells, can also be included in such analyses. It is predicted that a subpopulation of CD34<sup>+</sup>FGFR<sup>+</sup>Thy-1<sup>+</sup>VEGF-R<sup>+</sup> cells exist, which subpopulation can be identified and isolated by such systems by one of ordinary skill in the art without undue experimentation. This subpopulation represents a progenitor population capable of developing into either or both of the hematopoietic and endothelial lineages.

The isolated CD34<sup>+</sup>FGFR<sup>+</sup> cells grow exceedingly slowly in culture with a long lag of 4-6 weeks. The cells grow in an FGF-dependent manner, as shown in Example 3 and Table 5. A long dormant period is associated with a stem cell phenotype, indicating that these cells have growth characteristics compatible with stem cells.

While the CD34<sup>+</sup>FGFR<sup>+</sup> cells can be isolated in substantial purity, i.e., in a substantially homogeneous population, by the methods discussed above, such as, for example, by means of the FACS apparatus, it is not always necessary that the CD34<sup>+</sup>FGFR<sup>+</sup> stem cell population of the present invention be present in substantial purity. For example, for most purposes, it is sufficient if the population of cells contains greater than 90% of human stem cells characterized as CD34<sup>+</sup> and FGFR<sup>+</sup> or FGFR<sup>+</sup> with another indication of primitive phenotype. Other aspects of the present invention include subpopulations of the FGFR<sup>+</sup> primitive phenotype population which are substantially homogeneous for other markers. This includes the subpopulation of the FGFR<sup>+</sup> primitive phenotype human stem cells which are also positive for any one or more of the endothelial markers, any one or more additional primitive markers and/or any one or more of stromal cell markers, which

subpopulation is substantially homogeneous or is a composition wherein greater than 90% of said cells are FGFR<sup>+</sup> primitive phenotype and positive for one or more of those additional markers. Thus, the subpopulation may be a

5 substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and TIE-1<sup>+</sup>. The subpopulation may also be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or

10 CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and CD31<sup>+</sup> and/or TEK<sup>+</sup> and/or VEGFR<sup>+</sup> and/or VE-Cadherin<sup>+</sup> and/or positive for any other endothelial marker. Similarly, the subpopulation may be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and

15 positive for one or more of the other primitive markers, such as AC133, Thy-1 and c-kit. The subpopulation may also be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and also positive for one or more of the other

20 primitive markers and further positive for one or more of the endothelial markers. The subpopulation may also be a substantially homogeneous population or a composition in which greater than 90% of the cells therein are CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and also positive for one or more of stromal

25 markers, such as CD34<sup>+</sup>FGFR<sup>+</sup> (or CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>) and STRO-1<sup>+</sup>. Such subpopulations are also contemplated by the present invention.

With some of the utilities of the present invention, such as, for example, bone marrow transplantation,

30 the stem cell population may be a substantially smaller percent of the total cell count being administered. The remaining cells may be filler cells, which may be cells incapable of replicating. Alternatively, the remaining cells

may be any of the other types of cells from which the cells of the present invention are originally separated. Thus, for example, the present invention also comprehends populations containing at least 20% of any of the phenotypes of the present invention, i.e., CD34<sup>+</sup>FGFR<sup>+</sup>, CD34<sup>-</sup>lin<sup>-</sup>FGFR<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>Thy-1<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>TIE-1<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>CD31<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>VEGF-R<sup>+</sup>, CD34<sup>+</sup>FGFR<sup>+</sup>Thy-1<sup>+</sup>VEGF-R<sup>+</sup>, etc. Such a low purity subpopulation still defines over the prior art and yet maintains many of the advantages of the present invention for many of its proposed utilities. Compositions having greater than 30%, 40%, 50%, 60%, 70% or 80% of cells of any of the phenotypes of the present inventions are also considered to be part of the present invention.

Another way of defining the cellular compositions of the present invention is as a suspension of human cells, comprising pluripotent stem cells or endothelial stem cells which are substantially free of mature lymphoid and myeloid cells. Cells substantially all of which are of the FGFR<sup>+</sup> primitive phenotype are substantially free of mature lymphoid and myeloid cells.

Although the present invention has thus far been primarily described with respect to the preferred embodiment of CD34<sup>+</sup>FGFR<sup>+</sup> cells, it should be understood that FGFR<sup>+</sup> cells having other indications of primitive phenotype are also contemplated in accordance with the present invention. For example, CD34<sup>-</sup> cells which are also negative for lineage markers (lin<sup>-</sup>) may be even more primitive than CD34<sup>+</sup> cells (see Zanjani et al, 1999). Thus, the CD34<sup>-</sup>lin<sup>-</sup> phenotype is also considered to be an indication of primitiveness in accordance with the present invention. Additionally, when embryonic stem cells are used as the source of cells from which the population of the present invention is to be separated, all such cells, by definition, have a primitive

phenotype. Thus, FGFR<sup>+</sup> cells separated from an embryonic stem cell source will inherently be FGFR<sup>+</sup> cells with a primitive phenotype. Furthermore, other primitive markers, such as AC133, Thy-1 and c-kit, may also be used as markers for  
5 primitiveness. Thus, in its broadest aspect, the present invention relates to phenotypes which are FGFR<sup>+</sup>, as well as positive for any phenotype indicating primitive state cells, including, but not limited to, CD34<sup>+</sup>, CD34<sup>-</sup>lin<sup>-</sup>, being embryonic stem cells, AC133<sup>+</sup>, Thy-1<sup>+</sup> and c-kit<sup>+</sup>. The present  
10 invention further relates to subpopulations thereof as described above.

The pluripotent FGFR<sup>+</sup> primitive phenotype stem cells, or pluripotent stem cells of any of the other phenotypes of the present invention, have considerable  
15 commercial use, including one or more of the following:

(1) For bone marrow transplantation.

(2) To target delivery of anti-tumor agents.

Endothelial stem cells of the present invention can be used to target the delivery of angiostatic agents and anti-tumor  
20 agents to the rapidly proliferating vascular bed associated with tumors. Endothelial cells are long-lived, and the stem cells can be used as vectors to deliver angiostatic/antitumor agents to the rapidly expanding vascular bed associated with tumors without affecting the stable endothelium of  
25 established blood vessels.

(3) To coat valves and devices used in surgical procedures. The endothelial stem cells of the present invention can be used to coat valves and implant devices, eliminating many of the clotting problems currently  
30 associated with these devices. The endothelial stem cells can be cultured from specific individuals so that valves, implant devices, etc., may be coated with autologous endothelial cells. Panels of HLA-matched endothelial stem

cells can also be produced for these purposes.

(4) As vectors for genetic engineering.

Genetically engineered stem cells "homing" to the endothelium, bone marrow, or connective tissue stroma are  
5 long-lived and can secrete proteins, such as adenosine deaminase or clotting factors, as well as other proteins, such as t-PA, which promote thrombolysis. A wild-type gene can be incorporated into the endothelial stem cells, either by homologous or random recombination. With allogeneic  
10 endothelial stem cells, normal cells lacking the genetic defect can be used therapeutically. Other indications for gene therapy are introduction of drug resistance genes to enable normal stem cells to have an advantage and be subject to selective pressure, e.g., the multiple drug resistance  
15 gene. Disease other than those associated with endothelial cells may also be treated, where the disease is related to the lack of a particular excreted product, such as a hormone, enzyme, interferon, factor, or the like. By employing an appropriate regulatory initiation region, inducible  
20 production of the deficient protein is achieved so that production of the protein parallels natural production, even though production is in a different cell type from the cell type that normally produces such protein. A ribozyme, antisense or other message can be inserted to inhibit  
25 particular gene products or susceptibility to disease.

(5) To repair sites of vascular injury.

Engineered endothelial stem cells secreting such factors as tissue plasminogen activator, designed to help prevent restenosis after balloon angioplasty, can be infused.  
30 Asahara et al (1997) have demonstrated that endothelial stem cells selectively home to sites of vascular damage.

(6) To isolate new molecules that are important in stem and tumor cell biology.

(7) To form endothelium, stroma and blood cells, depending on the need at the time.

The stem cells of the present invention can be expanded in number by long-term *in vitro* culture with minimal differentiation until needed. However, the stem cells can produce blood cells when treated with the appropriate hematopoietic growth and differentiation factors, or form tubular network structures characteristic of endothelial cells upon treatment with the appropriate agents, or form fibroblast-like stromal cells upon treatment with the appropriate agents. Additionally, the cultured stem cells of the present invention can mature into functionally competent blood cells *in vivo*, capable of mediating antigen-specific immune responses, repopulating lympho-hematopoietic organs, and prolonging survival of animals with a destroyed hematopoietic system.

Although the phenotype of the particular stem cells isolated according to the present invention is new, one skilled in the art could readily, without undue experimentation, be able to use the stem cells of the present invention in the capacities described above, as well as in other capacities. Illustrations of such use are found in Wagner et al, U.S. Patent 5,744,347.

The following materials and methods are applicable to all of the following examples.

#### **Materials and Methods**

##### **Source of Human Cells**

Fresh or frozen cells from bone marrow, cytokine mobilized peripheral blood or cord blood were obtained from donors after informed consent.

Bone marrow or cord blood samples were diluted with 4 volumes RPMI 1640 medium containing 10% fetal calf serum (FCS). Mononuclear cells were separated on a

Histopaque®-1077 density gradient (Sigma Diagnostics, St. Louis, MO) and washed x 2 with PBS-citrate (PBS containing 13.6 mmol/L sodium citrate, 1 mmol/L adenosine and 2 mmol/L theophylline).

5                   Leukapheresis samples were not normally subjected to Histopaque®-1077 density gradient centrifugation but were washed X 2 with PBS-citrate before proceeding to the filtration step.

                  PBS-citrate washed cells (from all sources) were  
10   filtered through a 40 µm nylon cell strainer (Falcon 2340, Becton-Dickinson, New Jersey). Cells were resuspended in 2 ml PBS-citrate and overlaid on a 3 ml PBS-citrate/10% bovine serum albumin (BSA) cushion and then centrifuged for 10 minutes at 200 g at room temperature, to remove platelets  
15   (Thoma et al, 1994). This step was repeated once or twice.

                  If necessary DNase was used to digest DNA from cell debris.

#### Immunomagnetic Separation

                  Samples were enriched for CD34<sup>+</sup> cells by one of the  
20   following methods:

- (a)   magnetic activated cell sorting (MACS) columns, using anti-CD34 antibodies coated onto uniform, supermagnetic, polystyrene beads (Miltenyi Biotec, Auburn, CA).  
25       Separation was carried out on a MiniMACS device according to manufacturer's recommendation (Miltenyi Biotec, Auburn, CA).
- (b)   magnetic separation using the Dynal CD34 Progenitor Cell Selection System (Dynal A.S.,  
30       Oslo, Norway), which also uses anti-CD34 antibodies immobilized onto microbeads.

In both techniques magnetically labeled cells are separated



from other cells by means of a magnetic field.

#### Antibodies and Reagents

The following antibodies were used for immunofluorescent staining: CD34-FITC, CD34-PE, CD34-RPE-  
5 CY5, c-kit-PE, CD38-FITC, Mouse IgG-FITC, Mouse IgG-PE, Mouse IgG-RPE-Cy5, Mouse IgG2a, streptavidin-PE and goat-anti-mouse-RPE were purchased from Dako (Dako A/S, Glostrup, Denmark). Thy-1-FITC and Thy-1-PE were obtained from Immunotech (Immunotech, France). CD31-FITC, Mouse IgG  
10 Biotin, CD34-APC and Mouse IgG APC were obtained from Caltag (Caltag Laboratories, Burlingame, CA). HLA-DR-FITC, Mouse IgG and Goat IgG were purchased from Sigma (Sigma®, St. Louis MO) and CD34-APC, CD31-PE, Mouse IgG-APC from Becton-Dickinson (Becton-Dickinson, San Jose, CA). AC133-PE  
15 was obtained from Miltenyi (Miltenyi Biotec, Auburn, CA). VE-Cadherin-FITC was a kind gift from Dr. W. A. Muller, Cornell University, New York. TIE 1-FITC, TIE 1-PE, biotinylated TIE 1 and biotinylated TEK were generous gifts from Dr. T. Suda, Kumamoto University, Kumamoto, Japan.  
20 FGF-R1 antibody was obtained from Dr. W. L. McKeethan, Texas A and M University, Houston, TX or commercially, from QED Bioscience Inc. (San Diego, CA). Anti-FGF-R1-FITC was either purchased from QED or prepared in our laboratory. Anti-FGF-R1-APC was produced, purified and conjugated to  
25 allophycocyanin (APC) in our own laboratory. Conjugation of the antibody to APC was performed using a Phycolink™ conjugation kit, PJ25C, purchased from Prozyme (Prozyme, San Leandro, CA).

#### Cell Staining and Flow Cytometry

30 MACS-selected or Dynal-selected CD34<sup>+</sup> cells were resuspended in PBS/0.1% BSA/0.01% NaN<sub>3</sub>/Aprotinin 20µg/ml (PBS/BSA/N<sub>3</sub>/Aprotinin). Fc receptors and non specific

binding of immunoglobulins to cell surfaces were blocked with human IgG and either mouse or goat IgG where appropriate. Cells were incubated with appropriate antibodies for 30 minutes on ice. After washing x 2 with  
5 PBS/BSA/N<sub>3</sub>/Aprotinin, cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, San Jose, CA), equipped with an argon laser to excite FITC, PE and RPE-CY5 fluorochromes and a helium-neon diode, with time delay adjusted according to manufacturer's recommendations, for  
10 excitation of allophycocyanine (APC). 30,000 to 150,000 CD34<sup>+</sup> selected cells were analyzed using CellQuest software (Becton-Dickinson, San Jose, CA). The dye, 7-aminoactinomycin D (7-AAD) (Sigma, St. Louis, MO), was used in some experiments to identify dead cells. This was done  
15 to ensure that the CD34<sup>+</sup>FGFR<sup>+</sup> population was a viable population.

#### Cell Sorting

To isolate and study the growth characteristics of the CD34<sup>+</sup>FGFR<sup>+</sup> population, CD34<sup>+</sup> selected cells were obtained  
20 as previously described. These cells were incubated with antibodies to CD34 and FGF-R1 (as described above) and sorted on a Coulter Epics Elite (Beckman Coulter Inc., Fullerton, CA) into CD34<sup>+</sup>FGFR<sup>+</sup> and CD34<sup>+</sup> FGFR<sup>-</sup> populations. Sorted cells were incubated in a variety of media, including  
25 RPMI 1640,  $\alpha$ MEM, DMEM and long term culture medium (LTCM) (Myelocult H5100 from StemCell Technologies Inc., Vancouver, Canada) containing 12.5% horse serum and 12.5% FCS in the presence or absence of growth factors such as FGF-2 and VEGF. Growth was assessed by determining the number of  
30 cells present at various time points.

#### EXAMPLE 1: CD34<sup>+</sup>FGFR<sup>+</sup> Co-Expression of Other Antigens

CD34-enriched cells were purified from cytokine mobilized peripheral blood (PB), bone marrow (BM), or cord

blood (CB), using magnetic separation techniques (Dynal or MiniMacs). Fluorescent-labeled antibodies were used to assay for CD34<sup>+</sup>FGFR<sup>+</sup> cells and a percentage of CD34<sup>+</sup>FGFR<sup>+</sup> was determined for each experiment. The results of the fourteen experiments which were run are shown in Table 1. For the fourteen experiments, a mean of 4.4% ( $\pm$  2.3%) of CD34<sup>+</sup> cells expressed FGFR.

The presence of a third antigen on the CD34<sup>+</sup>FGFR<sup>+</sup> cells was also ascertained using fluorescent antibodies. The mean percentage of CD34<sup>+</sup>FGFR<sup>+</sup> cells expressing a particular third antigen is shown in Table 1 and is shown graphically in Figure 1. The error bars indicate the standard deviation (SD) and the numbers show the number of experiments assaying a particular antigen.

The experiments were run by submitting the CD34<sup>+</sup> or CD34<sup>+</sup>lin<sup>-</sup> enriched cells to the Becton-Dickinson FACSCalibur machine using the CellQuest System software for three- and four-color analysis. The techniques were similar to those described in "Flow Cytometry Analysis Using the Becton-Dickinson FACScan", Current Protocols in Immunology, unit 5.4, pages 5.4.1-5.4.19 (Supplement 16, 1995).

TABLE 1

		% CD34 <sup>+</sup> FGFR <sup>+</sup> Cells Expressing the Indicated Third Antigens										
Experiment #	Source	Selecton Method	% CD34 <sup>+</sup> FGFR <sup>+</sup>	CD38	HLA-Dr	AC133	Thy-1	c-kit	CD31	Tie 1	TEK	VE-Cadherin
1	PB	Dyn	3.2	100	43		97					
2	PB	Macs	7.9						66			
3	PB	Macs	3.6				50		38			
4	PB	Macs	3.1	100			38		85	29		
5	PB	Macs	3.9			50						
6	PB	Macs	6.6			67	26	94	99	23		
7	PB	Macs	3.6	96	53	73	57	40	97	47	25	85
8	PB	Macs	8.2			76	87				68	99
9	BM	Dyn	4.9				94	78	52			
10	BM	Macs	2.1				29	28	74			
11	CB	Dyn	5.8									
12	CB	Macs	1.3									
13	CB	Macs	6.5				31					
14	CB	Macs	1.1			55	23	67				
		Mean	4.4	99	48	64	52	57	70	33	47	86
		SD	2.3	2	7	11	28	27	23	12	30	12
		n	14	3	2	5	11	6	7	3	2	3

SD = Standard Deviation

n = Number

99% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express CD38 (range 96%-100%)  
 86% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express VE-Cadherin (range 75%-99%)  
 70% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express CD31 (range 38%-99%)  
 64% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express AC133 (range 50%-76%)  
 57% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express c-kit (range 28%-94%)  
 52% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express Thy-1 (range 23%-97%)  
 48% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express HLA-Dr (range 43%-68%)  
 47% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express TEK (range 25%-68%)  
 33% of CD34<sup>+</sup>FGFR<sup>+</sup> cells co-express Tie-1 (range 23%-47%)

It can be seen that in Experiment 7 results were obtained for third antigens from each of the panel of nine different antigens. The results of Experiment 7 are tabulated in Table 2. Furthermore, flow cytometry plots for Experiment 7 are shown in Figures 2A-2F. Figure 2A is a dot plot of forward scatter (FSC) versus side scatter (SSC) of all events. FSC gives an indication of cell size, i.e., low FSC equals small size. SSC gives an indication of cell granularity or complexity, i.e., low SSC equals low granularity. A region R1 is drawn to delineate cells of low-to-high FSC and low-to-medium SSC. This eliminates the area containing most of the cell debris and doublets.

Figure 2B is a histogram showing the intensity of staining with the dye 7-AAD (Philpott et al, 1996). 7-AAD is a large molecule which is not readily taken up by cells with intact cell membranes and which stains dead cells intensely. The histogram is gated on R1, and the region R2 is drawn to delineate live cells.

Figure 2C is a dot plot of SSC versus CD34 gated on R1 AND R2. The gates are set using Boolean logic in which the convention used for R1 AND R2 is  $R1 \cdot R2$ , and in this instance the gate delineates live cells in R1. The region R3 is drawn to delineate  $CD34^+$  cells in this plot. FITC was used to label the  $CD34^+$  cells.

Figure 2D is a dot plot of FSC versus SSC gated on R1 AND R2 AND R3 ( $R1 \cdot R2 \cdot R3$ ), i.e., this plot uses "backgating" to show the FSC/SSC characteristics of live  $CD34^+$  cells in R1.

Figure 2E is a dot plot of CD34 versus FGFR gated on R1 AND R2, i.e., gated on live cells in R1. The region R4 is drawn to delineate  $CD34^+FGFR^+$  cells.

Figure 2F is a dot plot of FSC versus SSC gated on R1 AND R2 AND R4 ( $R1 \cdot R2 \cdot R4$ ), i.e., this plot uses

"backgating" to show the characteristics of live CD34<sup>+</sup>FGFR<sup>+</sup> cells in R1. It is noted that the majority of live CD34<sup>+</sup>FGFR<sup>+</sup> cells have low FSC and low SSC, i.e., they are small cells with low granularity. The position of these cells on the  
5 scatter dot plot is somewhat unusual, as it appears in the region which is conventionally ignored.

**TABLE 2**  
**Experiment 7:**  
**Analysis of Live CD34<sup>+</sup>FGFR<sup>+</sup> Population from Cytokine Mobilized Peripheral Blood**

Number of CD34 <sup>+</sup> Cells	Number of CD34 <sup>+</sup> FGFR <sup>+</sup> Cells	% CD34 <sup>+</sup> FGFR <sup>+</sup> (% of CD34 <sup>+</sup> )	Phenotype	Number of Cells of Each Phenotype	Phenotype (% of CD34 <sup>+</sup> Population)	Phenotype (% of CD34 <sup>+</sup> FGFR <sup>+</sup> Population)
90121	4039	4.48	CD34 <sup>+</sup> FGFR <sup>+</sup> CD38 <sup>+</sup>	3876	4.30	96
104819	4829	4.61	CD34 <sup>+</sup> FGFR <sup>+</sup> HLA-DR <sup>+</sup>	2561	2.44	53
90803	2910	3.20	CD34 <sup>+</sup> FGFR <sup>+</sup> AC133 <sup>+</sup>	2106	2.32	73
111007	4773	4.30	CD34 <sup>+</sup> FGFR <sup>+</sup> c-kit <sup>+</sup>	1923	1.73	40
100291	2805	2.80	CD34 <sup>+</sup> FGFR <sup>+</sup> THY-1 <sup>+</sup>	1594	1.59	57
86441	2957	3.42	CD34 <sup>+</sup> FGFR <sup>+</sup> CD31 <sup>+</sup>	2869	3.32	97
8478	2956	3.50	CD34 <sup>+</sup> FGFR <sup>+</sup> VE-Cadherin <sup>+</sup>	2524	2.99	85
100148	3969	3.96	CD34 <sup>+</sup> FGFR <sup>+</sup> TIE <sup>+</sup>	1866	1.86	47
100758	2491	2.47	CD34 <sup>+</sup> FGFR <sup>+</sup> TEK <sup>+</sup>	686	0.68	28

**Mean: 3.64**  
SD 0.75

Mean % CD34<sup>+</sup> cells per sample = 98% ( $\pm 0.65$ )

Table 3 shows the results of some of the samples from Experiment 8. In these samples, CD34 staining was not included in order that other combinations of antigens could be assayed. Figure 3 shows these results graphically. One can see that all live FGFR<sup>+</sup>Thy-1<sup>+</sup> cells co-express VE-Cadherin as do 97% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells. Since 99% of CD34<sup>+</sup>FGFR<sup>+</sup> cells in Experiment 8 were found to be VE-Cadherin<sup>+</sup>, one can extrapolate from this experiment that the phenotype of the CD34<sup>+</sup>FGFR<sup>+</sup> population is CD34<sup>+</sup>FGFR<sup>+</sup>VE-Cadherin<sup>+</sup>Thy-1<sup>+</sup>AC133<sup>+</sup> and that approximately two-thirds are TEK<sup>+</sup>.

**Table 3**  
**Experiment 8: Analysis of Live FGFR<sup>+</sup> Cells from**  
**Cytokine Mobilized Peripheral Blood**

Sample #	Number of LIVE FGFR <sup>+</sup> Cells		Number of Cells of Each Phenotype	% of LIVE FGFR <sup>+</sup> Population	
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup>	609	96	
1	635	LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup>	534	84	1a
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup> THY-1 <sup>+</sup>	535	84	1b
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup>	550	93	
2	594	LIVE FGFR <sup>+</sup> AC133 <sup>+</sup>	316	53	2a
		LIVE FGFR <sup>+</sup> VE-CADHERIN <sup>+</sup> AC133 <sup>+</sup>	307	52	2b
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup>	270	76	
3	356	LIVE FGFR <sup>+</sup> AC133 <sup>+</sup>	198	56	3a
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup> AC133 <sup>+</sup>	181	51	3b
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup>	441	70	
4	627	LIVE FGFR <sup>+</sup> TEK <sup>+</sup>	399	64	4a
		LIVE FGFR <sup>+</sup> THY-1 <sup>+</sup> TEK <sup>+</sup>	269	43	4b

Table 3 shows that in this experiment:

All live FGFR<sup>+</sup>THY-1<sup>+</sup> cells co-express VE-Cadherin (1a, 1b)

About 97% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express VE-Cadherin (2a, 2b)

At least 91% of live FGFR<sup>+</sup>AC133<sup>+</sup> cells co-express THY-1 (3a, 3b)

At least 67% of live FGFR<sup>+</sup>TEK<sup>+</sup> cells co-express THY-1 (4a, 4b)

Example 2: CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> Co-Expression of Other Antigens

The following experiment was conducted using the techniques described for Example 1. Lineage depletion to obtain lin<sup>-</sup> cells was conducted as described in Bhatia et al,



1997, and Bhatia et al, 1998, using Dynal beads and magnetic separation. The results obtained are summarized in Table 4. It can be seen that 77% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express CD31, 40% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express c-kit, 47% of  
 5 CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express VE-Cadherin, and 18% of CD34<sup>+</sup>lin<sup>-</sup>FGFR<sup>+</sup> cells co-express TIE-1.

TABLE 4

Expt	CD34	% of CD34 <sup>+</sup> or CD34-LIN <sup>-</sup> FGFR <sup>+</sup> Cells Expressing Third Antigen	Cell Number
10			
	(a)	96% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are CD31 <sup>+</sup>	572
	-	77% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are CD31 <sup>+</sup>	211
		97% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are c-kit <sup>+</sup>	1500
15	-	40% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are c-kit <sup>+</sup>	107
	(b)	75% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are VE-Cadherin <sup>+</sup>	417
	-	47% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are VE-Cadherin <sup>+</sup>	570
20		42% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	237
	-	13% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	162
	(c)	37% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	1133
	-	23% of CD34 <sup>+</sup> LIN <sup>-</sup> FGFR <sup>+</sup> cells are TIE 1 <sup>+</sup>	625

#### 25 Example 3: Growth Characteristics

Cytokine mobilized peripheral blood cells were enriched for CD34<sup>+</sup> cells using magnetic separation. The cells were stained with fluorescent-labeled antibodies to CD34 and FGFR and sorted on a fluorescence-activated cell sorter  
 30 (FACS) into FGFR<sup>+</sup> and FGFR<sup>-</sup> populations. Cells were seeded at 1000 cells per well into collagen/gelatin coated wells containing long-term culture medium (LTCM) with either (a) no additions, (b) 10 ng/ml FGF-2 or (c) 10 ng/ml FGF-2 plus 10 ng/ml VEGF, and incubated at 37°C in a humidified incubator.  
 35 After 4-6 weeks, the number of cells per well were counted.

The results are shown in Table 5 and shown graphically in Figure 4. It can be seen that the FGFR<sup>+</sup> population grows in an FGF dependent manner and that the addition of VEGF together with FGF-2 further increases the growth of the cells.

**Table 5**  
**Cell Growth**  
(cells/well)

Expt	Population	ADDITIONS		
		None	FGF-2 (10 ng/ml)	FGF-2 + VEGF (both at 10 ng/ml)
(i)	CD34 <sup>+</sup> FGFR <sup>+</sup>	1734	11978	ND
	CD34 <sup>+</sup> FGFR <sup>-</sup>	1244	2500	ND
(ii)	CD34 <sup>+</sup>	1617	1457	2311
	CD34 <sup>+</sup> FGFR <sup>+</sup>	3227	8057	17140
	CD34 <sup>+</sup> FGFR <sup>-</sup>	1493	2098	3093

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references are entirely incorporated by reference herein, including all data, tables, figures and text present in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also incorporated by reference in their entirety.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the

invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, 5 such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, 10 materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention. Thus the expressions "means to..." and "means for...", or any method step language, as may be found in the specification above and/or in the claims 15 below, followed by a functional statement, are intended to define and cover whatever structural, physical, chemical or electrical element or structure, or whatever method step, which may now or in the future exist which carries out the recited function, whether or not precisely equivalent to the 20 embodiment or embodiments disclosed in the specification above, i.e., other means or steps for carrying out the same function can be used; and it is intended that such expressions be given their broadest interpretation.

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## WHAT IS CLAIMED IS:

1. A composition comprising a physiologically acceptable medium and human stem cells, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and also having another indicator of a primitive state.

2. A composition in accordance with claim 1, wherein said indicator of a primitive state is selected from the group consisting of CD34<sup>+</sup>, CD34<sup>-</sup>lin<sup>-</sup>, being an embryonic stem cell, Thy-1<sup>+</sup>, AC133<sup>+</sup> and c-kit<sup>+</sup>.

3. A composition in accordance with claim 2, wherein said indicator of a primitive state is CD34<sup>+</sup>.

4. A composition in accordance with claim 2, wherein said indicator of a primitive state is CD34<sup>-</sup>lin<sup>-</sup>.

5. A composition in accordance with claim 2, wherein said indicator of a primitive state is the state of being an embryonic stem cell.

6. The composition according to claim 1, wherein said human stem cells are selected from the group consisting of endothelial stem cells, stromal stem cells and hematopoietic stem cells.

7. The composition according to claim 1, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more of the markers Thy-1<sup>+</sup>, AC133<sup>+</sup> and c-kit<sup>+</sup>.

8. The composition according to claim 1, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

9. A composition in accordance with claim 8, wherein said markers indicative of endothelial cells are



selected from the group consisting of TIE-1<sup>+</sup>, TEK<sup>+</sup>, CD31<sup>+</sup>, VE-Cadherin<sup>+</sup> and VEGFR<sup>+</sup>.

10. The composition according to claim 1, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

11. A composition in accordance with claim 10, wherein said marker indicative of stromal cells is STRO-1<sup>+</sup>

12. The composition according to claim 7, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

13. The composition according to claim 7, wherein greater than 20% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

14. A composition in accordance with claim 1, wherein said human stem cells are a subpopulation of peripheral blood cells, bone marrow cells, or cord blood cells.

15. A composition in accordance with claim 1, wherein said human stem cells are a subpopulation of embryonic stem cells.

16. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and also having another phenotype indicative of a primitive state.

17. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and

further having one or more of the markers Thy-1<sup>+</sup>, AC133<sup>+</sup> and c-kit<sup>+</sup>.

18. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

19. A composition in accordance with claim 18, wherein said markers indicative of endothelial cells are selected from the group consisting of TIE-1<sup>+</sup>, TEK<sup>+</sup>, CD31<sup>+</sup>, VE-Cadherin<sup>+</sup> and VEGFR<sup>+</sup>.

20. The composition according to claim 1, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

21. A composition in accordance with claim 20, wherein said marker indicative of stromal cells is STRO-1<sup>+</sup>

22. The composition according to claim 7, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of endothelial cells.

23. The composition according to claim 7, wherein greater than 90% of said cells are human stem cells characterized as FGFR<sup>+</sup> and either CD34<sup>+</sup> or CD34<sup>-</sup>lin<sup>-</sup> and further having one or more markers indicative of stromal cells.

24. A composition in accordance with claim 16, wherein said human stem cells are a subpopulation of peripheral blood cells, bone marrow cells, or cord blood cells.

25. A composition in accordance with claim 16,

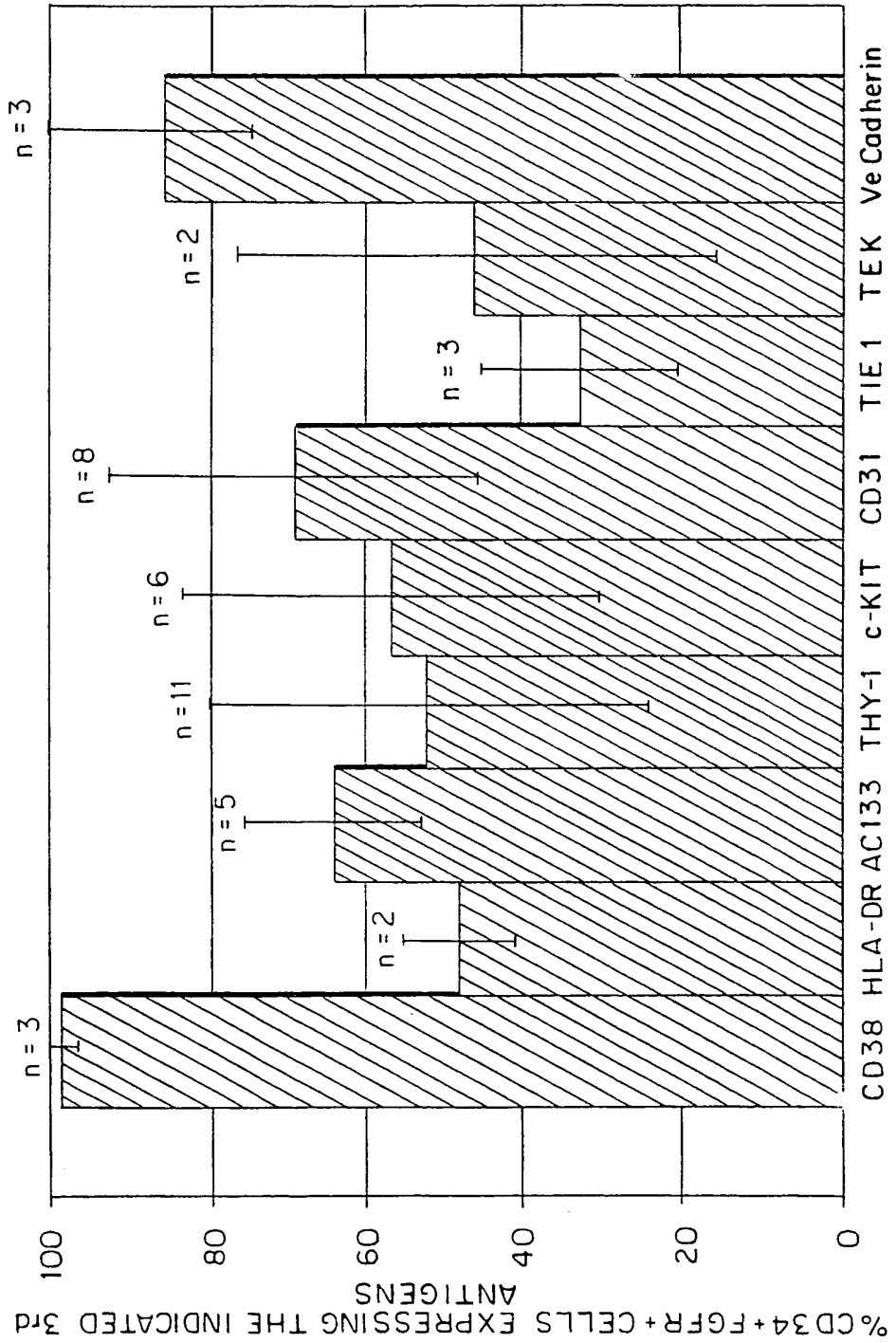
wherein said human stem cells are a subpopulation of embryonic stem cells.

26. A cellular composition comprising a substantially homogeneous population of cultured human stem cells displaying a phenotype of FGFR<sup>+</sup> and another phenotype indicative of a primitive state, which cells are capable of giving rise to cells selected from the group consisting of endothelial cells, stromal cells and hematopoietic cells.

27. A suspension of human cells comprising pluripotent stem cells substantially free of mature lymphoid and myeloid cells.

28. The suspension according to claim 27, wherein said stem cells are selected from the group consisting of endothelial cells, stromal cells and hematopoietic cells.

FIG. 1



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FIG. 2A

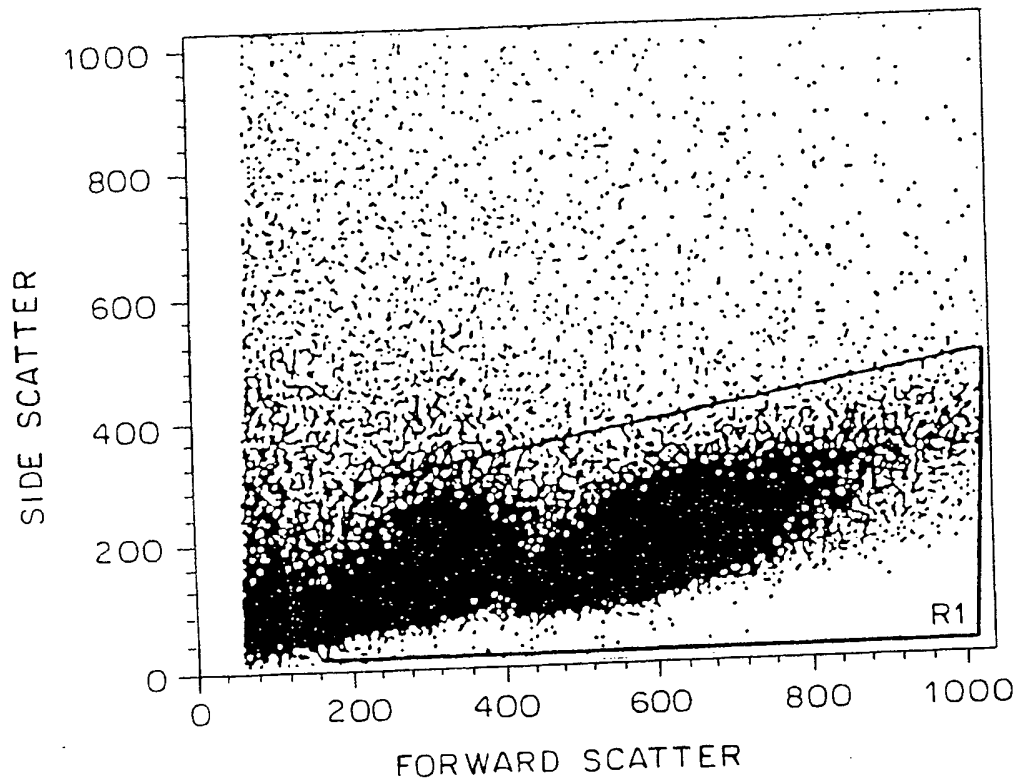
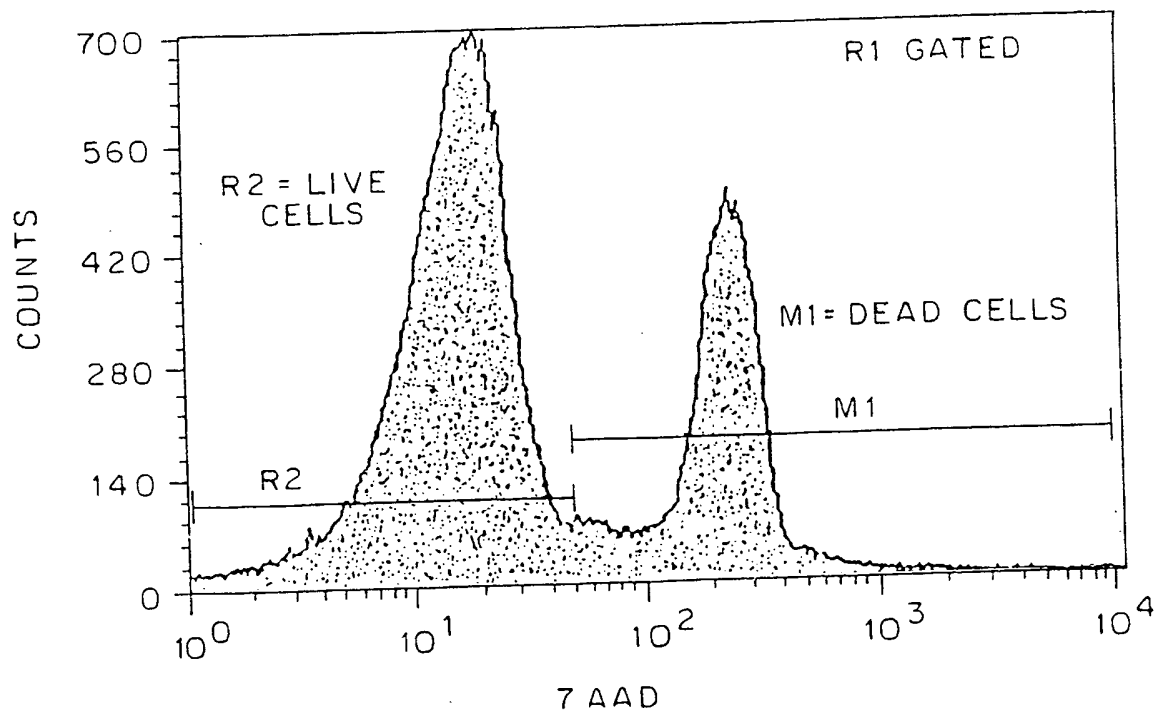


FIG. 2B



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FIG. 2C

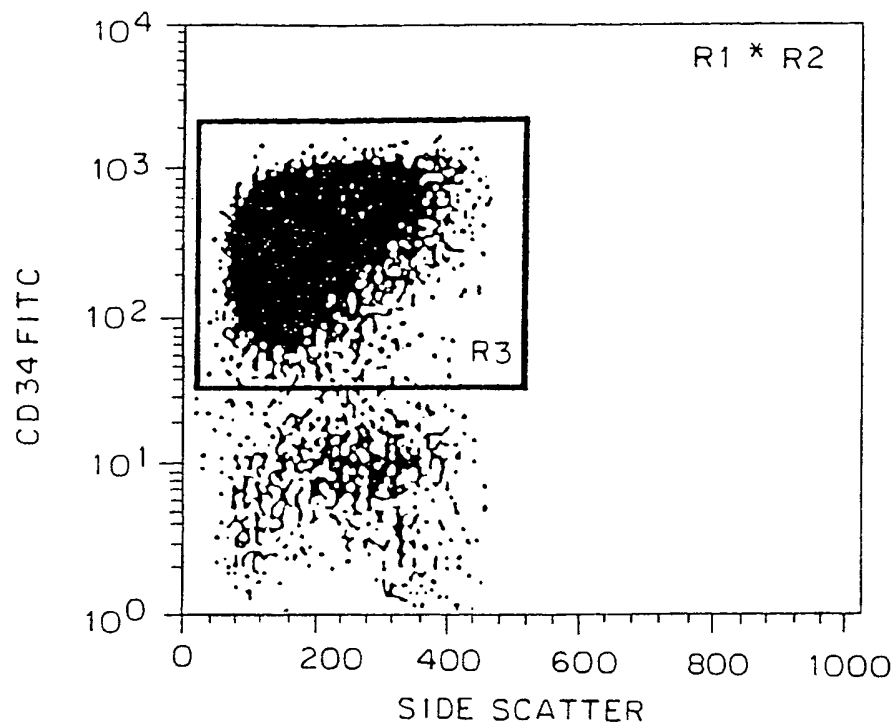
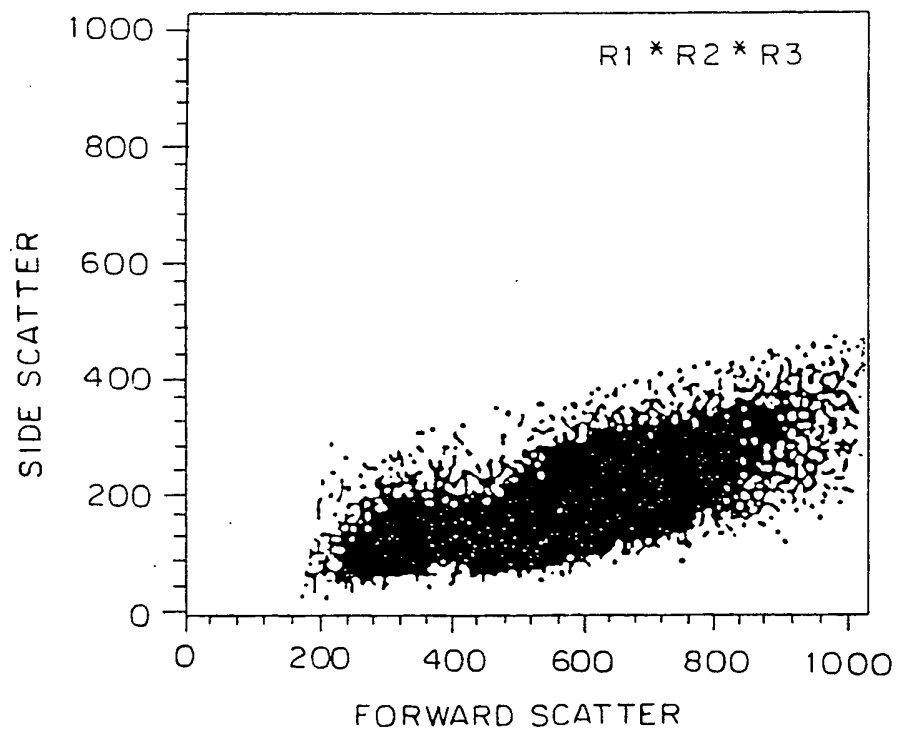


FIG. 2D



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FIG. 2E

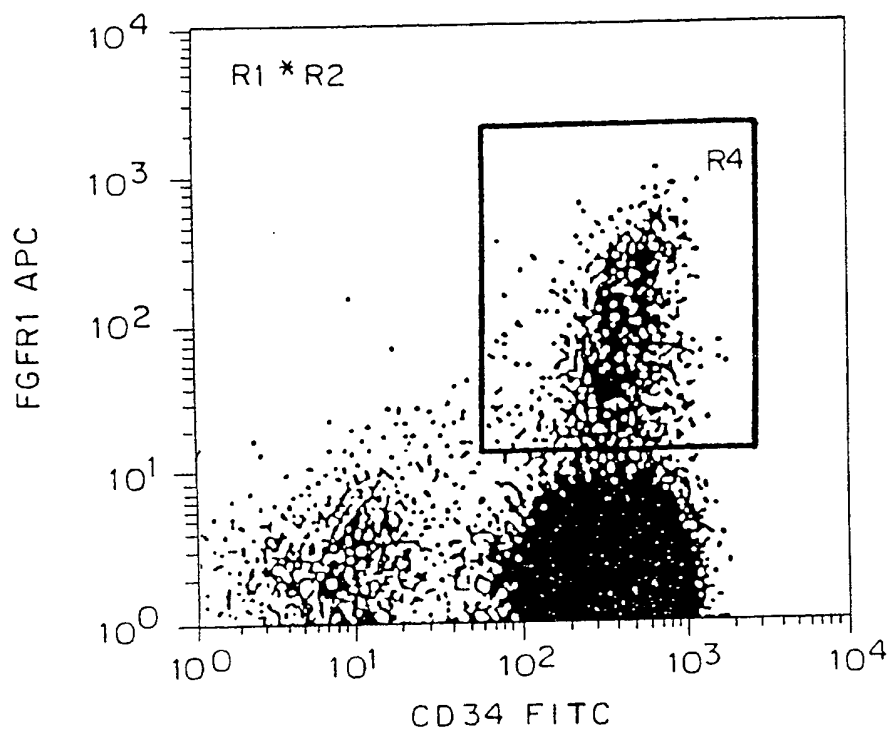


FIG. 2F

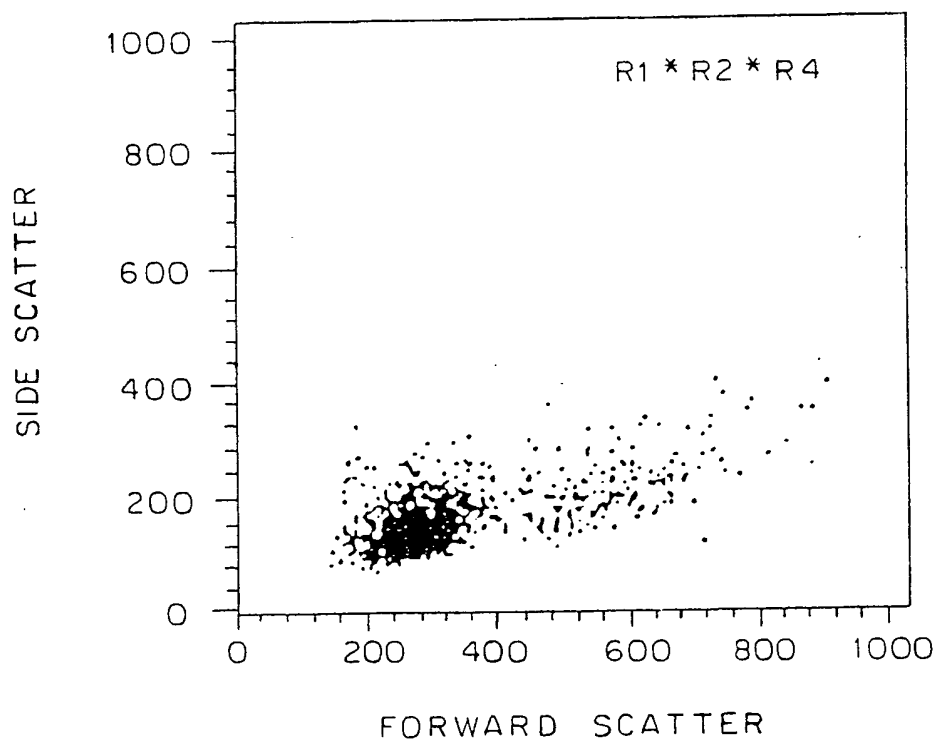


FIG. 3

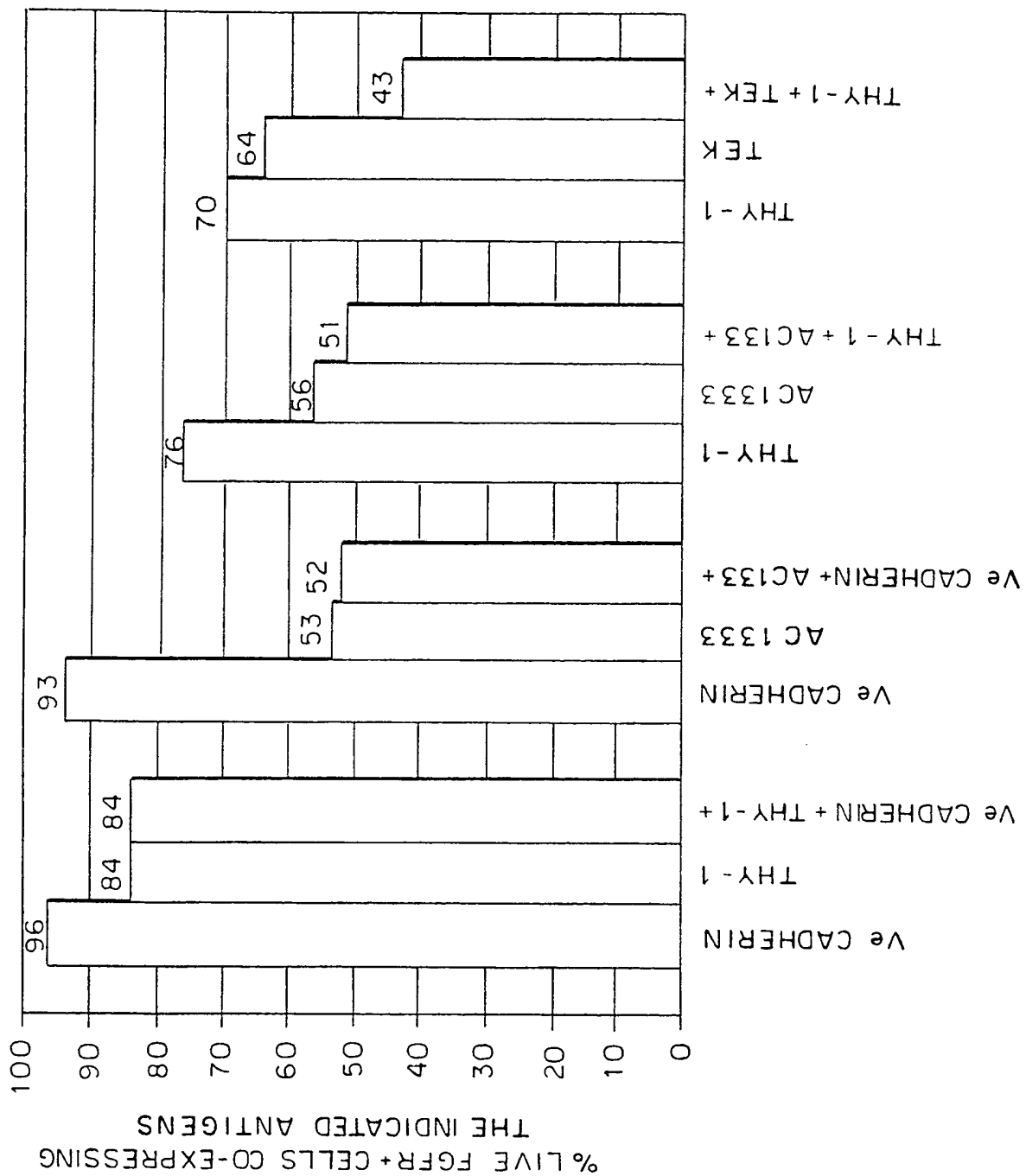
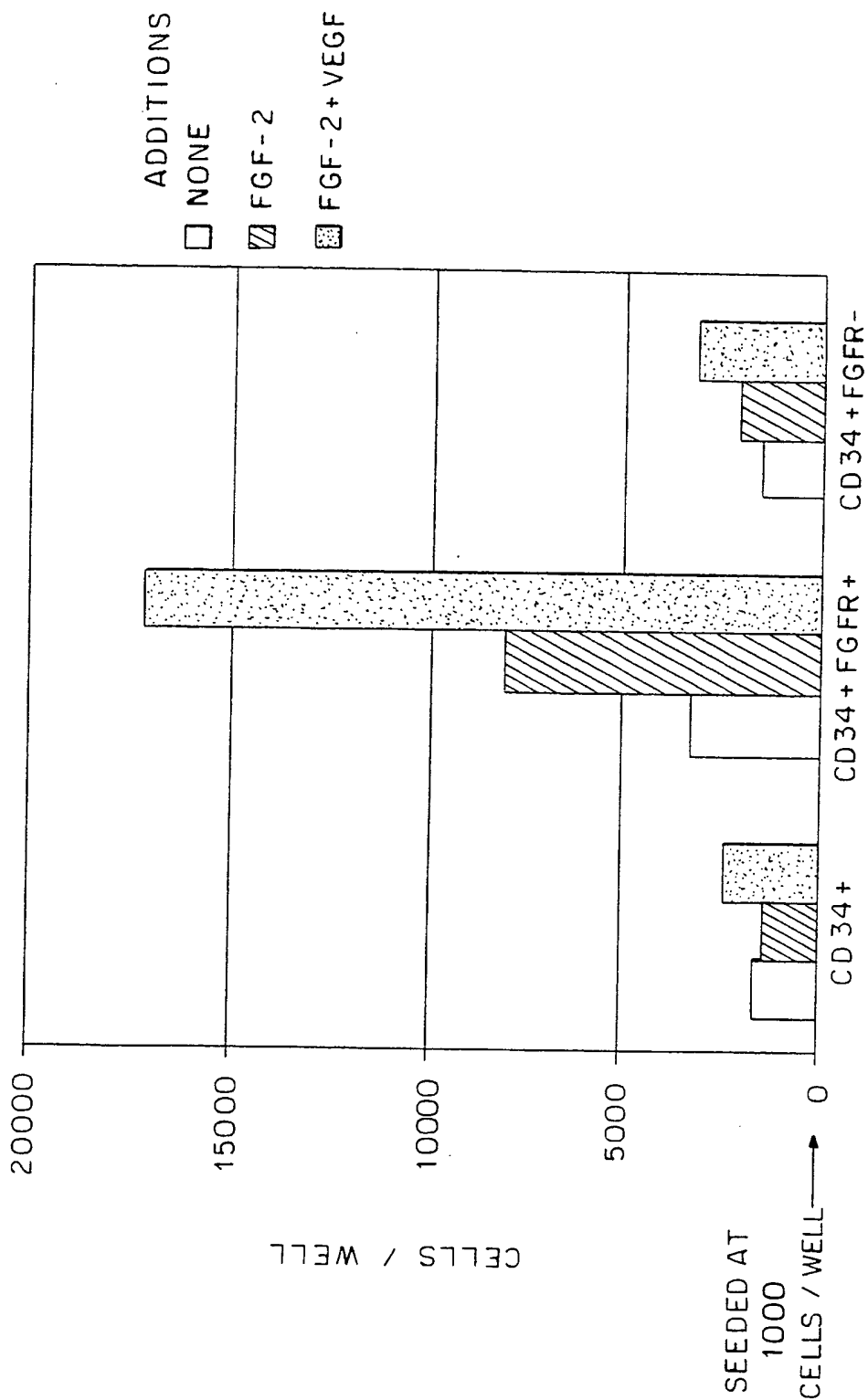




FIG. 4



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/US 99/19730

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N5/08 A61K35/14 A61K35/28 A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	REDWINE J M ET AL: "In situ expression of fibroblast growth factor receptors by oligodendrocyte progenitors and oligodendrocytes in adult mouse central nervous system." JOURNAL OF NEUROSCIENCE RESEARCH, (1997 OCT 15) 50 (2) 229-37. , XP000891229 the whole document	1-28
A	RATAJCZAK MARIUSZ Z ET AL: "Effect of basic (FGF-2) and acidic (FGF-1) fibroblast growth factors on early haemopoietic cell development." BRITISH JOURNAL OF HAEMATOLOGY 1996, vol. 93, no. 4, 1996, pages 772-782, XP000891218 ISSN: 0007-1048 the whole document	1-28
	-/-	



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

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- "P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search

20 March 2000

Date of mailing of the international search report

04/04/2000


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Moreau, J

## INTERNATIONAL SEARCH REPORT

In  Application No  
PCT/US 99/19730

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 690 926 A (HOGAM B.L.M.) 25 November 1997 (1997-11-25) the whole document	1-28
P,X	BURGER P E ET AL: "Isolation and characterization of a CD34+ population that expresses fibroblast growth factor receptors." BLOOD, 40TH ANNUAL MEETING OF THE AMERICAN SOCIETY OF HEMATOLOGY; MIAMI BEACH, FLORIDA, USA; DECEMBER 4-8, 1998, vol. 92, no. 10 SUPPL. 1 PART 1-2, 15 November 1998 (1998-11-15), page 56A XP000891230 ISSN: 0006-4971 the whole document	1-28

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/19730

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5690926 A	25-11-1997	US 5453357 A US 5670372 A	26-09-1995 23-09-1997